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On the Estimation of Alginic Acid in Seaweeds by the Method of Colloid Titration

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Received October 16, 1957

This method is based on the theory that positive polymer ions combine stoichiometrically with negative ones in a dilute solution at a definite pH value. A definite amount of 0.005 N solution of a positive colloid like N-trimethylated glycol chitosan is added to a solution or an extract (pH ca. 7) containing alginic acid, the excess of the added positive colloid being titrated with a standard solution (0.0025 N) of a negative one such as potassium salt of polyvinyl sulfonate. The end-point of this titration is indicated by the color change of toluidine blue or the sudden precipitation of reactants. Whenever an extract contains some protein, however, it is necessary to digest this protein by treating the extract with pepsin prior to titration. The amounts of alginic acid can be calculated from the value, which is obtained by deducting the results of several titrations from the mean-titre of blank experiments.

Several methods for the purpose of estimation of alginic acid in seaweeds have been reported, but in most cases they are laborious and inadequate for application of a large number of samples. Of these methods, the gravimetric method[1] has perhaps been used more frequently than the others for routine work, but this method is very laborious and difficult to be applied on a microscale on account of the colloidal properties of alginic acid. On the other hand, though the method of carbon dioxide evolution[2] would seem to be the most specific for the estimation of polyuronide materials, it is inconvenient to be applied to a large number of samples. Recently, Percival and his co-workers[3] have reported a colorimetric method based on the reaction of alginic acid with carbazole and sulfuric acid, yet this is practically of little use.

Therefore, as a more practicable method the author has tried to apply the same method (the method of colloid titration) as that one used successfully for the estimation of pectin[4], lignin[5] and etc. in plant materials to alginic acid.

Terayama[6] has studied on the stoichiometric combinations of various polymer ions and the chemical mechanisms of metachromasy, which is a phenomenon of the color change of a dye combined with polymer ions, from which he has advocated a new volumetric analysis of polymer ions, called the method of colloid titration. In his studies, macramin (N-polymethylated chitosan) was used as the standard positive colloid, and potassium salt of polyvinyl sulfonate (henceforth called PVSK) as the negative.

This stoichiometric combination of oppositely charged polymer ions is fundamentally due to the Coulombic electrostatic attraction, which is proportional to their degree of polymerisations. Therefore, the degree of polymerisation of the standard positive polymer ion, which combines with the objective negative one or PVSK at a colloid titration, should be as great as possible. When the degree of polymerisation is smaller, the electrostatic attraction between these positive and negative ions gets weaker. Accordingly, the combination among both polymer ions does not take place stoichiometrically and the sudden precipitation of reactants does not clearly occur at an equivalent point, considerably lowering the accuracy of the titration. This has also been suggested by Deuel and his co-workers[7].

---

[1] Agricultural Chemical Laboratory, Kyoto University, "Experimental Textbook of Agricultural Chemistry", Sangyo Tosho Press., 1950, p. 1014.

[2] R.M. McCready, H.A. Swenson and W.D. Maclay, *Ind. Eng. Chem.*, Anal. Bd., **18**, 290 (1946).

[3] E.G.V. Percival and his co-workers, *J. Soc. Chem. Ind.* (London), **67**, 161, 420 (1948).

[4] S. Okimasu, *Bull. Agr. Chem. Soc. Japan*, **20**, 29, 49 (1956).

[5] R. Senju, *Bull. Chem. Soc. Japan*, **25**, 131 (1952), **26**, 143, 148 (1953).

[6] H. Terayama, *Chem. Research* (in Japanese), **1**, 75 (1948), **4**, 31 (1949); *J. Poly. Sci.*, **8**, 243 (1952); *Arch. Biophys.*, **50**, 55 (1954).

From this point of view, the degree of polymerisation of macramin is too small. (It is ca. 20 according to Terayama.) The author, therefore, has used N-trimethylated glycol chitosan (henceforth called MGC), first prepared through the deacetylation and methylation of glycol chitin[8] by the author, as the standard positive colloid in place of macramin, and it was found that the estimation of alginic acid by colloid titration was possible.

This report deals with some experimental results concerning the applicability of this method, also stating the general procedures.

EXPERIMENTAL

1. Materials. (1) Reagents used for colloid titration*. The same MGC and PVSK were also used in this experiment as in the case of pectin estimation reported previously[4]. From this, it is clear that their equivalent molecular weights per one ionized group are 383.6 (MGC) and 165.4 (PVSK), respectively. Toluidine blue (0.1% aqueous solution), used as the indicator in the colloid titration, was a Merk product. (2) Na-alginate. This was prepared from the commercial product as follows: Na-alginate was precipitated from the solution and washed repeatedly with 90% ethanol, dehydrated with acetone, and dried in a vacuum desicator. The purity of this sample in uronic anhydride proved to be 96.95% by the method of carbon dioxide evolution[2].

2. Colloid Titration Curve of the Na-Alginate Solution. A quantity of 0.4950 g of Na-alginate was dissolved in water and the solution was filled up to 500 ml with water, each 5-ml of the solution being placed in several Erlenmeyer-flasks (capacity 100 ml). Then, to each flask 20 ml of distilled water and 2 ml of the aqueous solution of acetic acid or ammonia differing from each other in their concentrations were added, in order to adjust the pH of each titration medium. Ten ml of the 0.005 N MGC solution and two drops of 0.1% toluidine blue as an indicator were added further and shaken. After being stood for a few minutes, a 0.0025 N PVSK solution was added to each flask titrimetrically through a semimicro-burette (capacity 15 ml), until the blue color of the indicator changed into reddish-purple. At this point, the flocculation or the precipitation of reacting complexes appeared abruptly. The color change of indicator was easily detected with the aid of a light of a tungsten lamp. The pH of each reaction medium was measured at the end of each titration.

On the other hand, control titrations were conducted using 5 ml of distilled water in place of Na-alginate solution. The difference between both titration results at the same pH was evaluated in all cases. (These differences indicate the ml of a 0.0025 N PVSK solution, corresponding to the amounts of MGC combined with Na-alginate contained in 5 ml of the sample solution, and are called the MGC combining capacity.)

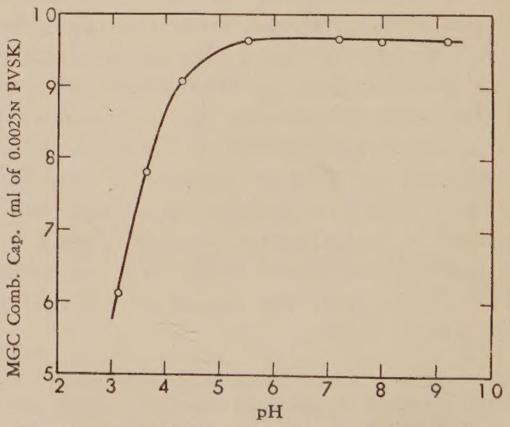

FIG. 1. Colloid Titration Curve of Na-Alginate Solution.

The MGC combining is the value for 5 ml of the Na-alginate solution (0.0960 g/100 ml).

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[7] H. Deuel, J. Selnis and A. Denzler, *Helv. Chim. Acta*, **26**, 1671 (1953).

[8] R. Senju and S. Okimasu, *J. Agr. Chem. Soc. Japan* (in Japanese), **23**, 432 (1950).

* These reagents are now on sale by Seiko Chemical Drugs Co. (Hakozaki Showa Machi, Fukuoka, Japan).

The relation between these MGC combining capacities and the pH of each reaction medium, namely the colloid titration curve, is shown in Fig. 1.

3. Colloid Titration Curves of Several Seaweed Extracts. Sample seaweeds (*Laminaria japonica* Areschoug, *Eisenia bycyclis* Setchell and *Undaria pinnatifida* Suringer) were dried materials, which were finely cut, washed lightly with water for several times and dehydrated in a vacuum desicator. About 2 g of each sample was weighed accurately, and immersed in in 50 ml of a 0.1 N chlorine solution for about one hour, then the liquid was decanted off. After being treated repeatedly in the same manner until bleaching was almost accomplished, it was washed free from chlorine with water. Alginic acid was extracted by treating this material with 250 ml of 1% sodium carbonate or 0.6% sodium oxalate warmed at about 80° under constant stirring, until the whole mass was dissolved almost completely. The extract was then filtered and washed for several times with hot water, the trasparent filtrate being filled up to 500 ml with water.

Portions of 5-ml of the filtrate were placed in several Erlenmeyer-flasks, and neutralized with 0.05 N hydrochloric acid (at Na-carbonate extract) or 0.05 N sodium hydroxide (at Na-oxalate extract). Then, to each flask 2 ml of the aqueous solution of acetic acid or ammonia differing from each other in their concentrations and 20 ml or a less amount of distilled water were added, in order to adjust the pH of each titration medium and equalize the whole volume. Thereafter, following the same procedures as mentioned in the colloid titration of Na-alginate solution, relations between both MGC combining capacities and the pH of each titration medium were obtained as shown in Fig. 2.

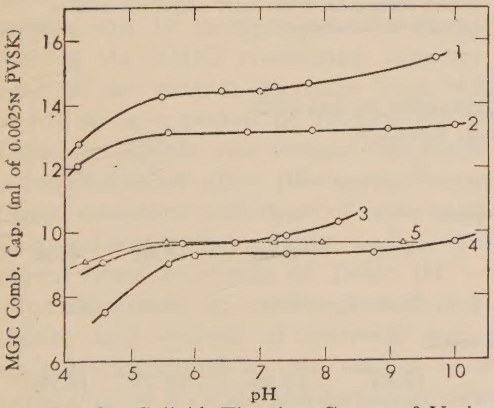

FIG. 2. Colloid Titration Curves of Various Seaweed Extracts.

The MGC combining capacity is the value for 5 ml of each extract. The numerals shown in above figure are extract nos. in Table I, but curve 5 indicates the colloid titration curve of the Na-alginate solution.

4. Digestion of the Protein Contained in Seaweed Extracts with Enzyme. 100 ml of the extract (*Laminaria japonica* Areschung, extractive solvent: 1% Na-carbonate) mentioned above was neutralized with 21.0 ml of 0.2 N hydrochloric acid, and an aliquot was offered to enzymic digestion as follows: (1) Digestion with pepsin. Each 5 ml of the neutralized extract was placed in several Erlenmeyer-flask, and to each flask 5 ml of a HCl-KCl buffer solution (pH 1.8) and 2 ml of a 0.3% pepsin solution (dissolved in the same buffer) were added and shaken. After being incubated at 40° for a definite period, it was neutralized exactly with dilute ammonia and titrated in the same manner as described above. In this case also, control titration was carried out using 5 ml of distilled water in place of extract. Changes of MGC combining capacities due to incubating hours are shown in Table II. (2) Digestion with pancreatin. All procedures were carried out according to the same manner as in the pepsin digestion, except that a phosphate buffer (pH 8.4) and a 0.3% pancreatin solution pretreated with a small amount of enterokinase were used in place of HCl-KCl buffer and pepsin solution. In this case, however, the incubated solution was neutralized with dilute acetic acid. These experimental results are also shown in Table II.

DISCUSSION

Both MGC and PVSK are strong electrolytes of high molecular weight, of which the degree of ionization does not change along with the pH of the solution. On the other hand, as alginic acid is the polymer of mannuronic acid, its degree of ionization is supposed to decrease at the acidic side of pH. Accordingly, a reasonable pH range of the solution would be necessary for the stoichiometric combination among both alginic acid and MGC.

As shown in Fig. 1, MGC combining capacity of the Na-alginate solution is constant at pH levels above approximate 6.0, but it falls suddenly at pH lower than this, undoubtedly indicating the decrease of the ionization degree of alginic acid. Thus, the content of alginic acid must be evaluated from the constant value of MGC combining capacity, which is proved to be 9.64 ml in Fig. 1. This indicates that if the equivalent molecular weight of Na-alginate is assumed as the theoretical value ($C_6H_7O_6Na$: 198) the concentration of the solution is 0.0954 g/100 ml, which coincides well the value of 0.0960 g/100 ml calculated from the weight and the purity of the sample. Thus, it is clear that the content of alginic acid in a solution can be estimated from MGC combining capacity at pH 7∼8 on the basis of the theoretical equivalent molecular weight (176) of alginic acid.

The colloid titration curves of several seaweed extracts also have a close resemblance to that of Na-alginate solution as shown in Fig. 2, except that curves have a tendency to ascend slightly to the alkaline side which is sometimes observed. In this case, the MGC combining capacity of each of these extracts at pH 7.0 and their content of alginic acid calculated from each value are shown in Table. I. From these results it is understood that the alginic acid content estimated by this method using the extract with 1% sodium carbonate is considerably larger than those estimated by the gravimetric or the carbon dioxide evolution method, but in the case of the extract with 0.6% sodium oxalate the

TABLE I

ESTIMATION RESULTS OF ALGINIC ACID BY THE COLLOID TITRATION METHOD

Extract No.	Sample Used	Extractive Solvent	Results of Colloid Titration		Content of Alginic Acid (%)	
			MGC Comb. Cap. (ml of 0.0025 N PVSK)	Content of Alginic Acid (%)	Gravimetric Method	Carbon Dioxide Evolution Method
1	*Undaria pinnatifida* Suringer, 1.9620 g	1% Na_2CO_3	14.45	32.4	25.7	24.2
2	*Laminaria japonica* Areschoug, (A), 2.0664 g	〃	13.15	28.0	21.9	22.0
3	*Eisenia bycyclis* Setchell, 1.7710 g	〃	9.70	24.1	19.8	18.2
4	*Laminaria japonica* Areschoug, (A), 1.9670 g	0.6% Na-Oxalate	9.28	20.8	20.5	22.0

Notes: (1) Extracts were filled up to 500 ml, and MGC combining capacities were obtained using each 5-ml of the extracts. (2) In the gravimetric method, alginic acid was precipitated from 100 ml of the extracts with 1N H_2SO_4, the content being found by deduction of the weight of protein estimated by the nitrogen content of the precipitate from weight of the latter. In the case of the carbon dioxide evolution method, about 0.2 g of the dried sample was used in all cases, and other procedures were the same as already reported by McCready and co-workers[2].

TABLE II

ENZYMIC DIGESTION OF PROTEIN CONTAINED IN SEAWEED (*Laminaria japonica* Areschoug) EXTRACT

Reaction Time (Hours)	0	0.5	1	2	5	9	22
			Digestion with Pepsin				
MGC Comb. Cap. (ml)	10.87	10.52	10.20	9.70	8.88	8.62	8.58
Content of Alginic Acid (%)	28.0	27.1	26.3	25.0	22.9	22.2	22.1
			Digestion with Pancreatin				
MGC Comb. Cap. (ml)	10.94	10.87	10.90	10.83	10.83	10.75	10.68
Content of Alginic Acid (%)	28.2	28.0	28.1	27.9	27.9	27.7	27.5

Notes: 100 ml of extract No. 2 listed in Table I was neutralized with 21.0 ml of a 0.2N HCl, and MGC combining capacities were obtained using each 5-ml of the solution. The content of alginic acid is shown on a dried-material basis.

TABLE III

ESTIMATION OF ALGINIC ACID IN SEVERAL SEAWEEDS

Species of Sample	Extractive Solvent	Treatment with Pepsin	Content of Alginic Acid (%)		
			Colloid Titration	Gravimetric	Carbon Dioxide Evolution
Undaria pinnatifida Suringer	1% Na_2CO_3	—	32.4	25.7	24.2
〃	〃	20 Hrs. at 40°	25.2	—	〃
〃	0.6% Na-Oxalate	—	22.9	23.3	〃
Laminaria japonica Areschoug, (B)	1% Na_2CO_3	—	23.7	18.2	19.5
〃	〃	20 Hrs. at 40°	19.0	—	〃
〃	0.6% Na-Oxalate	—	18.5	—	〃
Eisenia bycyclis Setchell	1% Na_2CO_3	—	24.1	19.8	18.2
〃	〃	20 Hrs. at 40°	18.5	19.2	〃
〃	0.6% Na-Oxalate	—	16.9	17.4	〃
Hijikia fusiforme (Harvey) Okamura	1% Na_2CO_3	20 Hrs. at 40°	17.3	18.9	18.1
〃	0.6% Na-Oxalate	—	16.5	—	〃

content is somewhat smaller than those obtained by these methods. This is considered to be due to the presence of some protein contained in the extract with 1% sodium carbonate, besides, when 0.6% sodium oxalate is used as an extractive solvent the extraction seems to be incomplete.

Consequently, digestion of the protein by treating the extract with enzyme, namely pepsin or pancreatin, was attempted. As shown in Table. II, it can hardly be digested with pancreatin, this being considered to be attributed to the fact that alginic acid interferes the action of trypsin. Whereas, the protein can be easily digested with pepsin, that is, the MGC combining capacity for 5-ml of the extract decreases from 10.87 ml to 8.58 ml in a period of twenty-two hours. Thus, the alginic acid content estimated by colloid titration after this pepsin treatment almost coincided with those of other methods. This problem is above doubt, as it is already known from the results of Table. III.

In conclusion, it was confirmed that the alginic acid content of seaweeds was conveniently estimated by the colloid titration method, using a 1% sodium carbonate solution as an extractive solvent and digesting some protein contained in the extract with pepsin prior to titration.

GENERAL PROCEDURES

About one or two gram of finely cut dried seaweeds is used for the estimation. This sample is weighed accurately, and immersed in 50 ml of 0.1 N chlorine solution for about one hour at the room temperature, then the liquid is decanted off. After being treated repeatedly in the same manner until bleaching is almost accomplished, it is washed free from chlorine with water. Alginic acid is extracted by treating this material with about one hundred and twenty-five times of a 1% sodium carbonate solution warmed at about 80° under constant stirring, until the whole mass is dissolved almost completely. The extract is then filtered and washed for several times with hot water, the transparent filtrate being filled up to 250 or 500 ml with water. By the use of a small amount of the extract, the presence of some protein is tested. If protein is contained, digestion with pepsion is carried out in the following manner: Five ml of the extract is placed in an Erlenmeyer-flask, and it is neutralized with 0.1 N hydrochloric acid. Then, 5 ml of a HCl-KCl buffer solution (pH 1.8) and 2 ml of a 0.3% pepsin solution (dissolved in the same buffer) are added, shaken and incubated at 40° for about twenty hours. In this case, the amount of the added pepsin solution and incubating hours are not always constant, but differ according to the activity of the pepsin. After this treatment it is neutralized with dilute ammonia, then 20 ml of distilled water, 10 ml of a 0.005 N MGC solution and two drops of 0.1% toluidine blue are added further and shaken. After being stood for a few minutes, a 0.0025 N PVSK solution is added titrimetrically through a semimicro-burette, until the blue color of the indicator changes into reddish-purple. This color change is easily detected in most cases with the aid of a light of a tungsten lamp. However, when it is almost undetectable by the color change, the end-point of this titration can be conveniently detected by the sudden precipitation of reacting complexes. The pH of the titration medium is necessary to be 7~8 at the end of titration. Several titrations are operated with the same extract, and the average is taken. On the other hand, a control titration is made using 5 ml of distilled water in place of the extract. Thus, the content of alginic acid in the sample seaweed is estimated from the difference between both titration results, namely, the MGC combining capacity, several estimation examples being shown in Table III. (One ml of the 0.0025 N PVSK solution corresponds to 0.44 mg of alginic acid.) This method can be conveniently applied on a microscale and is also applicable for a large number of samples.

The author wishes to express his thanks to Dr. R. Senju of Kyushu Universtity for his kind suggestions and constant encouragement throughout the course of this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 2, p. 68~77, 1958]

Effect of Gamma-Ray upon Food Microorganisms

Studies Concerning Gamma-Ray Resistivity of *Escherichia coli* (I)

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Department of Agricultural Chemistry, Tokyo University of Education

Received October 21, 1957

The author has carried out a study concerning a simplified method for the measurement of gamma-ray resistivity of *E. coli*. This report deals with comparison of each survival curve of 11 species of bacteria and the influence of addition of chemicals on a medium containing *E. coli* by gamma-ray irradiation by application of this method.

INTRODUCTION

Ever since X-ray was discovered, much study has been undertaken in respect to the effect of radio-active rays against microorganisms. During the past ten years, studies regarding the relation between the radio-active ray and microorganisms has progressed to a considerable extent, attributed to the possibility of utilization of a new food preserving method, the so-called "Cold Sterilizatlon".

Proctor has conducted studies concerning the action of X-ray produced at a high voltage on finely ground beef and observed that the bacterial counts were considerably reduced as the result of irradiation[1]. The author has also recognized that microorganisms have entirely disappeared by radiation with a high dose gamma-ray in packaged fishcake and raw tuna fish meat[2,3]. Brasch has conducted experiments on the lethal effect of non-sporeforming bacteria by using a powerful electron beam produced from Capacitron and has reported the perfect lethal dose of *E. coli*, *A. aerogenes*, and other bacteria[4]. The author has conducted research on the survival-curve with gamma-ray irradiation on several kinds of bacteria and has reported that the survival-curve can even be obtained by using a small amount of cobalt-60 provided the bacteria were suspended in a phosphate buffer solution[5]. Dunn has studied on the relation between the non-sporeforming bacteria and gamma-ray, and has compared gamma-ray resistivity on various kinds of bacteria applying the survival-curve and data of perfect lethal dose[6]. Proctor has conducted experiments on the lethal effect of different kinds of bacteria by the kilo-curie of cobalt-60 and has made a comparison determinating the survival-curves of 11 species of bacterial strains and their survival ratios as shown on the semi-long paper. The survival-curve turned out to be almost a straight-line[7].

There are also many other reports concerning the survival-curves and lethal doses the use of radio-active ray radiation. These reports can be divided into two categories; i.e., the first group considering the experimental plots graphed from the survival-curve should be shown in one single line, whereas, the other group considers experimental plots to be actually existing because they are a record of living cell bacteria. Consequently, the survival-curve should not be a single line but a group of plots between acute-angled lines. Dunn has investigated the lethal effect of various types of microorganisms using electron beam and gamma-ray, reported the existence of a certain range of lethal dose applicable for all types of bacteria[6]. The survival-curve drawn by Proctor, as previously mentioned, was a line drawn on the section where the points were most densely populated. Edward has studied on the lethal effect of cathod-ray upon spores of *B. subtilis* and has discovered that a survival-curve obtained from a solution with a certain concentration was more or less the same as those at other concentrations[8].

The condition, in which the bacteria are maintained has effect on the survival ratio and also the lethal dose, thus resulting in different lines on the graph. By observing the resulting lines, the influence of such change of condition can be recognized. Proctor has added pea-peree in medium containing *E. coli*[9], Edward has added salt and milk[8], Stapleton has added all kinds of chemical compounds[10], and studied the influence of change of condition by comparison of the change of survival-curves.

The author has modificated and discovered a simplified testing method for investigation of the influence of different conditions affecting *E. coli*, stated below.

---

[1] B.E. Proctor, and H. Fram, Effect of X-ray irradiation on the bacterial count of ground meat, Research Reports on Quartermaster Contract Projects from July 1, 1942, to June 30, 1943, by M.I.T., p 217.

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[9] S.A. Goldblith, B.E. Proctor, S. Davison and E.M. Oberle, How Processing Conditions Affect Microorganism Radioresistance, *Nucleonics*, **13**, 42-45 (1955).

[10] G.E. Stapleton, A.J. Sbarra and A. Hollander, Some Nutritional Aspects of Bacterial Recovery from Ioning Radiation, *J. Bact.*, **70**, 7-14 (1955).

EXPERIMENTAL

(1) Bacterial Strains Used.

The following strains were used. The majority of the strains used here included *Escherichia coli* (YCI), one of the cultures stocked at the School of Medicine, Keio University. Occasionally, other strains which were isolated from foods by the author[2)3)], or from stock cultures the University of Tokyo were used. Among the other strains there were 3 strains of *Alcaligenes*, one each of *Escherichia*, *Aerobacter*, *Serratia* and *Achromobacter*, all isolated from fish cake and raw tuna fish meat, and 2 strains of *Pseudomonas* and one strain of *Proteus*, which were obtained from the stock cultures of the Univ. of Tokyo.

(2) Experimental Method.

(a) Samples for irradiation tests; All strains were incubated for 24 hours at 37°C and their biological characteristics were checked in advance of each experiment.

The original suspension was made in a phosphate buffer solution at pH 7.2 at the concentration of 1 mg/ml (10^3 cell/ml), after the organisms were centrifuged and washed two or three times. The original suspension was diluted by ordinary serial ten-fold dilution method. The final ten-fold dilution was made in a dissolved nutrient agar (NISSAN SUISAN K.K. pH 7.2) which was kept at 40°C. An amount of exactly one-cc of the mixture was pipetted into a sterile petri dish, and it was then solidified. The thickness was 1.5 mm, when one-cc of the above mixture was pipetted into each dish. In this experiment, thin and small petri dishes, each differing in thicknesses but having the same diameter of 30 mm were specially used. They were divided into two sets, each set consisting of two dishes, the dish-thicknesses being 7.8 mm and 12.8 mm in the first set, and 8.0 mm and 13.0 mm in the second set. These dishes were specially made small and thin (30 mm diameter) according to the following reasons;

1. In order to radiate the maximum number of samples with multi-Curie of cobalt-60.
2. To minimize the error of irradiation dose on the samples by keeping mediums of one-cc under 1.5 mm.

The concentrations of samples used in Experiment 1 were 10^7 cell/ml, 10^5 cell/ml, 10^4 cell/ml, 10^3 cell/ml and 10^2 cell/ml respectively. In Experiments 2 and 3, the 10^2 cell/ml solid was used.

TABLE I
RELATIVE DISTANCE OF SAMPLES FROM RADIATION SOURCE

Sample No.	Distance cm	
	Experiment 1, 3.	Experiment 2.
1	5.5	4.5
2	6.3	5.3
3	7.1	6.1
4	7.9	6.8
5	8.7	7.6
6	9.5	8.4
7	10.3	9.2
8	11.2	10.0
9	12.5	11.2
10	13.8	12.5

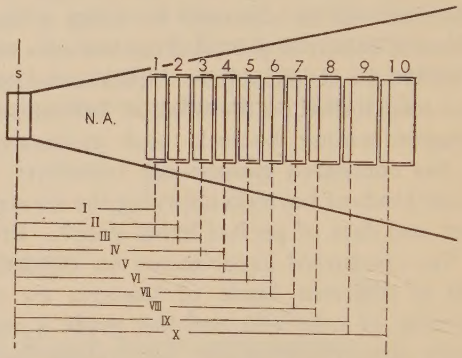

FIG. 1. Relation between Radiation Source and Sample used in γ-ray Experiment.

S: Cobalt-60 40 Curie
1~10: Sample No.
I~X: Distance between Source and Samples
N.A.: Nutrient agar containg bacteria

(b) Irradiation method; The radiation source was cobalt-60, 40 curie, preserved at the School of Medicine, Keio University. The position of samples in the irradiation beam is shown in Figure 1 and also in Table I. All samples were irradiated at room temperature within the range of 19°C to 27°C. By the use of an air-conditioner, room temperature was maintained at this certain temperature allowing a tolerance of ±1°C. The time of irradiation in Experiment 1, was from 2 to 20 hours, in Experiment 2, 1.5 hours, and Experiment 3, 2 hours.

(c) Method for calculation of bacteria; In order to determine the bacterial count of non-irradiated and irradiated samples, the bacteria were divided into two groups. Both groups were equally treated except for the irradiation procedure. Both non-irradiated and irradiated samples were put into an incubator at 37°C immediately after irradiation, and cultured for 24 hours. All colonies developed were counted by means of a binocular microscope, using a weak magnification.

The definition of the survival ratio is defined as the percentage obtained from the bacterial count of irradiated and non-irradiated samples. The calculation of radiation dose is based on the quantity of cobalt-60, distance between the samples and the radiation source, and irradiation time. As the distance and radiation time is usually constant, the quantity of cobalt-60 which gradually decays, determining the radiation dose. Two kinds of dosimetry were used to figure the quantity of cobalt-60 several times during the period of one year. A Röntgen-Meter was used for physical dosimetry, bio-assay using the irradiated sperm of Sea-Chestnut in case of biological dosimetry[11]. The dose calculation of the interval of several tests was based on the fact that cobalt-60 decays at the rate of 1.1% per month.

When the concentration of non-irradiated samples exceeds 10^2 cell/ml, it is impossible to count all the colonies on a single plate. Therefore, the controls are diluted and poured into 10^2 cell/ml dishes. The colonies are then counted and the bacterial count in the initial concentration is thus estimated.

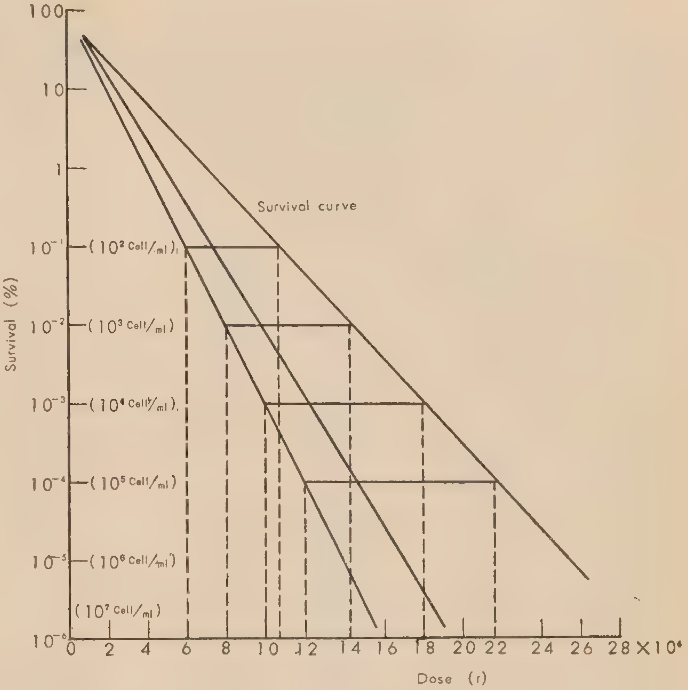

FIG. 3. Range of lethal dose of *E. coli* (YCI) by γ-ray irradiation at the range of various bacterial concentration per ml.

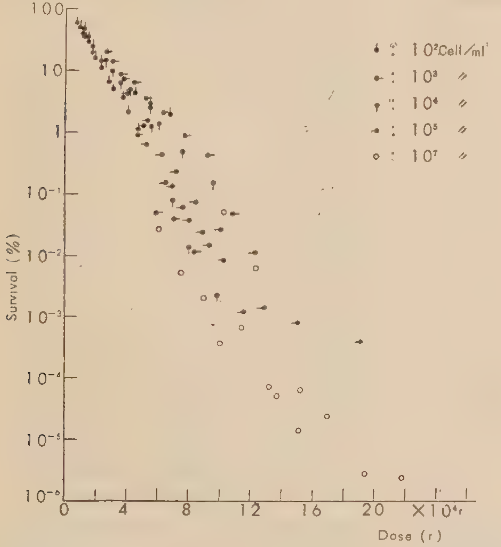

FIG. 2. Survival Ratio of *E. coli* (YCI) by γ-ray Irradiation at the Range of Various Bacterial Concentration per ml.

RESULTS AND DISCUSSION

(1) Experiment 1

E. coli (YCI) was used in this experiment. Bacterial concentrations of the samples were 10^7, 10^5, 10^4, 10^3 and 10^2. In order to perform as many determinations as possible the bacterial count of petri dishes among the irradiated samples, the time of irradiation was regulated within 10 to 20 hours. Depending upon the time of irradiation, in some dishes no colony was detectable. Thereby, an apparent adequate lethal dose was observed.

The survival ratios obtained from various irradiation doses using a concentration of 10^2 cell/ml are illustrated in Table II, 10^3 cell/ml in Table III, 10^4 cell/ml in Table IV, 10^5 cell/ml in Table V, and 10^7 cell/ml in Table VI respec-

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[11] H. Yamashita, J. Fujita and W. Watanabe, On the Physical and Biological Dosimetries of the Depth Dose for the Teletherapy of Cobalt-60 Multicurie Unit, *Jap. Radio-Isotopes*, **4**, 1 (39-44) (1961) (in Japanese).

Table II

Survival Ratio of *E. coli* (YCI) by γ-Ray Irradiation at the Range of 10^2 count per ml.

Rad. time hr	Dose r	Bacterial count/ml	Survival %
0	0	4.7×10^2	100
3	47.385	5	1.1
3	36.108	17	3.6
3	28.431	29	6.2
3	22.983	73	15.5
3	18.954	85	18.0
3	15.873	97	20.6
3	13.506	134	28.5
3	11.421	178	37.8
3	9.192	215	45.8
3	7.677	236	50.2
0	0	5.4×10^2	100
2.5	39.488	9	1.7
2.5	30.090	28	5.2
2.5	23.693	63	11.7
2.5	19.153	99	18.3
2.5	15.795	132	24.4
2.5	13.228	185	36.1
2.5	11.255	218	40.3
2.5	9.518	240	44.4
2.5	7.660	290	53.7
2.5	6.398	308	57.0
0	0	4.4×10^2	100
7	83.314	0	0
7	65.317	9	2.1
7	53.025	13	3.0
7	43.736	17	3.9

Table III

Survival Ratio of *E. coli* (YCI) by γ-Ray Irradiation at the Range of 10^3 count per ml.

Rad. time hr	Dose r	Bacterial count/ml	Survival %
0	0	3.9×10^3	100
10	156.190	0	0
10	119.020	2	0.051
10	93.710	17	0.43
10	75.750	36	0.92
10	62.480	81	2.1
10	52.320	136	3.5
10	44.510	219	6.3
10	37.640	316	8.1
10	30.300	495	13.0
10	25.300	730	19.0
0	0	8.7×10^3	100
16	98.864	0	0
16	82.784	1	0.011
16	70.432	3	0.034
16	59.568	4	0.046
16	47.952	91	1.04
16	40.032	637	7.3

Table IV

Survival Ratio of *E. coli* (YCI) by γ-Ray Irradiation at the Range of 10^4 count per ml.

Rad. time hr	Dose r	Bacterial count/ml	Survival %
0	0	1.7×10^4	100
8	124.950	6	0.035
8	95.216	25	0.15
8	74.968	90	0.53
8	60.600	237	1.4
8	52.544	401	2.4
8	41.856	721	4.2
8	35.608	1015	5.9
8	30.112	1717	10.0
8	24.240	2527	15.0
8	20.240	3040	18.8
0	0	9.6×10^4	100
18	110.222	0	0
18	93.132	2	0.002
18	79.014	12	0.013
18	53.946	76	0.079
18	45.036	2560	2.7

Table V

Survival Ratio of *E. coli* (YCI) by γ-Ray Irradiation at the Range of 10^5 count per ml.

Rad. time hr	Dose r	Bacterial count/ml	Survival %
0	0	2.4×10^5	100
16	249.904	0	0
16	190.432	1	0.0004
16	149.936	2	0.0008
16	121.200	28	0.011
16	99.968	65	0.026
16	83.712	184	0.074
16	71.216	492	0.20
16	60.224	1108	0.44
16	48.480	3077	1.23
16	40.480	9450	3.8
0	0	3.3×10^5	100
17	159.309	0	0
17	128.775	5	0.0015
17	106.216	30	0.0090
17	88.944	86	0.026
17	75.667	214	0.064
17	63.988	443	0.13
17	51.510	2134	0.64
0	0	1.8×10^5	100
18	168.678	0	0
18	136.350	1	0.0005
18	115.024	2	0.0011
18	94.176	19	0.016
18	80.118	70	0.039
18	67.752	235	0.13
18	54.540	2613	1.5

TABLE VI

SURVIVAL RATIO OF *E. COLI* (YCI) BY γ-RAY IRRADIATION AT THE RANGE OF 10^7 COUNT PER ML.

Rad. time hr	Dose r	Bacterial count/ml	Survival %
0	0	2.7×10^7	100
16	252.720	0	0
16	192.576	1	0.000003
16	151.632	15	0.00006
16	122.576	1737	0.0064
16	101.088	14560	0.054
0	0	3.7×10^7	100
18	281.142	0	0
18	214.236	1	0.0000027
18	168.678	9	0.000024
18	136.350	19	0.000051
18	112.464	281	0.00075
0	0	3.7×10^7	100
20	187.420	0	0
20	151.500	5	0.000016
20	124.960	21	0.000066
20	104.640	119	0.00037
20	89.020	684	0.0021
20	75.280	1765	0.0055
20	60.600	8944	0.028

tively. The apparent adequate lethal dose in each case was slightly different from each another, and in case of the concentration of 10^2 cell/ml, it was in range of 6.5×10^4 and 8.3×10^4 r, in case of 10^3 cell/ml, either $11.9\times10^4-15.6\times10^4$ r or $8.3\times10^4-9.9\times10^4$ r, in case of 10^4 cell/ml, $9.3\times10^4-11.1\times10^4$ r, and in case of 10^5 cell/ml, $19.0\times10^4-25.0\times10^4$ r, $12.9\times10^4-15.9\times10^4$ r, or $13.6\times10^4-16.9\times10^4$ r and in case of 10^7 cell/ml, $19.3\times10^4-25.3\times10^4$ r, $21.4\times10^4-28.1\times10^4$ r, or $15.2\times10^4-18.7\times10^4$ r.

When figures in Table II to VI were recorded on a semi-log paper, as in Figure 2 the mass of points showed the survival-curve which was almost a straight-line. The masses of these points are scattered in a wider range when a higher bacterial concentration is used. This tendency is identical with the apparent adequate lethal dose previously mentioned. In other words, the mass of these points are inclosed in an acute-angled triangle. (Figure 3)

The main cause of this divergence is considered to be attributed the gamma-ray resistant organisms originally contained in the strain or caused by those which were mutagenetically created during irradiation.

As shown in Figures 2 and 3, the survival-curve of *E. coli* always assumes a same straight-line, inspite of different concentrations of bacteria used.

Comparison among the survival-curves, in the past, were only made with highly concentrated medium. As previously mentioned, the survival-curve derived from high concentrated mediums shows a wider range than those of low concentrations. Therefore, any comparison between two different survival-curves with such a wider range should be inaccurate in this experiment. Consequently, in order to compare the gamma-ray resistivity against various types of bacteria or the influence of a different condition on a certain bacterial strain more accurately, the narrow-portion of the survival-curve should be employed. However, if the concentration of 10^1 cell/ml is taken, difference of bacterial count in each dish would be too great, and the percentage of its tolerance also too large resulting to be unpractical. Thus, the concentration of 10^2 cell/ml was decided to be the most suitable for this experiment.

(2) Experiment 2

Besides *E. coli*, 10 strains of bacteria; i.e., *E. intermedium*, *Alcaligenes faecalis*, *Al. bookeri*, *Al. metalcaligenes*, *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Ps. fluorescens*, *Serratia plymuthicum*, *Achromobacter aquamarius*, and *Proteus vulgaris* were used.

The radiation temperature employed here was room temperature, in the range of 19° C and 27° C, the radiation time being 1.5 hours.

The result is as shown in Table VII, if survival-curves of each of the strains mentioned above are illustrated in the same semi-long paper, they are as shown in Figure 4. In this particular experiment, the apparent adequate lethal dose cannot be determined. Therefore, in order to compare the gamma-ray resistivity of each strain, the angle of

TABLE VII

SURVIVAL RATE OF VARIOUS TYPES OF BACTERIA BY γ-RAY IRRADIATION

E. coli		*A. aerogenes*		*E. intermedium*		*Ac. aquamarius*		*Se. plymuthicum*	
Dose r	Survival %	Dose r	Survival %	Dose r	Survival %	Dose r	Survival %	Dose r	Survival %
4.0×10^3	89.0	4.2×10^3	79.3	4.8×10^3	44.7	4.2×10^3	77.2	4.2×10^3	52.4
6.3	64.4	5.6	—	6.6	33.5	5.6	54.0	5.6	47.2
9.1	39.0	7.8	45.5	9.6	24.9	7.8	33.4	7.8	31.3
14.4	26.6	11.8	32.7	15.1	7.6	11.8	15.2	11.8	15.2
26.2	8.0	19.9	22.9	27.4	1.0	19.9	4.2	19.9	5.2

Al. faecalis		*Al. bookeri*		*Al. metalcaligenes*		*Pr. vulgaris*		*Ps. aeruginosa*	
Dose r	Survival %	Dose r	Survival %	Dose r	Survival %	Dose r	Survival %	Dose r	Survival %
4.3×10^3	40.5	3.4×10^3	50.4	4.8×10^3	40.0	3.4×10^3	48.3	3.4×10^3	32.5
6.4	27.9	5.4	27.5	6.6	24.7	5.3	38.6	5.3	21.8
9.2	12.6	7.6	—	9.6	12.9	7.5	21.9	7.5	10.2
14.6	8.0	11.4	9.5	15.1	6.5	11.3	11.6	11.3	4.9
26.5	—	19.2	2.8	27.4	0.6	19.0	5.6	19.0	1.5

Ps. fluorescens	
Dose r	Survival %
3.4×10^3	32.0
5.3	15.9
7.5	6.0
11.3	1.1
19.0	0

—: contaminated noncountable.

each survival-curve or the lethal dose value obtained from a certain survival ratio must be taken. In this case, the certain survival ratio in question is 10%. If the survival ratio chosen is below 10%, tolerance would be too high as gamma-ray resistant organisms are involved or attributable to the experimental error of bacterial counting. The lethal dose value obtained from the 10% survival ratio was the same as that of 90% of lethal dose value (LD 90). LD 90 would be better in this experiment, and those values obtained from each survival-curve are shown in Table VIII. When all the LD 90 values are given on the same table, the gamma-ray resistivity of each strain is comparable with each other.

TABLE VIII

RELATION BETWEEN γ-RAY SENSITIVITY OF VARIOUS TYPES OF BACTERIA

Strains	LD 90 value $\times10^4$ r
Escherichia coli	2.20
Aerobacter aerogenes	2.15
Escherichia intermedium	1.40
Achromobacter aquamarius	1.30
Serratia plymuthicum	1.30
Alcaligenes faecalis	1.25
Proteus vulgaris	1.20
Alcaligenes bookeri	1.10
Al. metalcaligenes	1.10
Pseudomonas aeruginosa	0.80
Ps. fluorescens	0.60

(3) Experiment 3

In Experiment 2, comparison of radio resistivity between each type of bacteria, can be made by using the method as previously mentioned.

In this experiment studies were made concerning the influence of the addition of chemical substances to a medium containing only a single strain of *E. coli*. The chemical

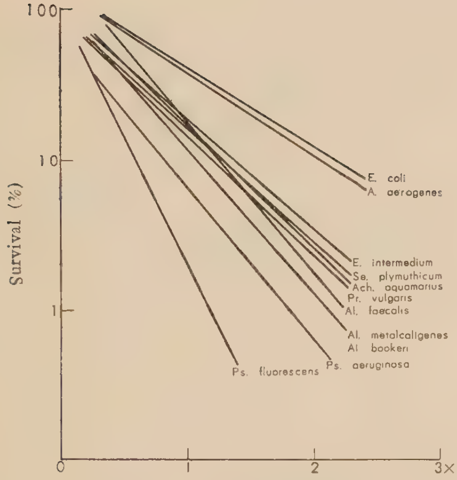

FIG. 4. Effect of γ-ray Irradiation on Survival of Dilute Suspension of Various Types of Bacteria.

TABLE IX
SURVIVAL RATIO OF *E. COLI* (YCI) BY γ-RAY IRRADIATION IN NUTRIENT AGAR CONTAINED SUCROSE

Sucrose	Average Survival Percentage		
Sucrose	0%	0.5%	1.0%
concentration	1223 cells/ml	836 cells/ml	725 cells/ml
Dose $\times 10^3$ r			
24.6	8.4%	10.9%	9.4%
15.7	20.9	21.0	24.6
10.8	38.1	32.9	33.9
7.8	49.3	46.3	47.0
5.2	66.3	62.2	61.4
Sucrose	2.0%	4.0%	8.0%
concentration	723 cells/ml	740 cells/ml	1059 cells/ml
Dose $\times 10^3$ r			
32.3	4.0%	5.4%	5.4%
19.4	16.4	12.0	19.9
12.9	29.7	32.1	30.0
9.2	41.3	43.3	43.7
6.3	51.9	51.9	58.9

TABLE X
SURVIVAL RATIO OF *E. COLI* (YCI) BY γ-RAY IRRADIATION IN NUTRIENT AGAR CONTAINED NaCl

	Average Survival Percentage		
NaCl	0%	0.5%	1.0%
concentration	868 cells/ml	743 cells/ml	948 cells/ml
Dose $\times 10^3$ r			
24.9	8.0%	9.8%	11.2%
15.8	21.6	24.6	25.5
10.9	36.5	40.2	43.0
7.9	41.4	44.5	50.7
5.3	56.4	62.1	65.1
NaCl	2.0%	3.0%	4.0%
concentration	923 cells/ml	606 cells/ml	650 cells/ml
Dose $\times 10^3$ r			
32.7	6.0%	8.4%	4.3%
19.6	21.4	22.3	19.6
13.1	34.9	39.1	31.4
9.3	45.0	51.4	47.7
6.3	63.9	59.3	60.1

substances added, were sucrose, salt and thiourea.

Sucrose, of a pure quality useable as chemical reagent, was added to the culture medium in amounts of 0.5, 1.0, 2.0, 4.0 and 8.0%. Nacl, also of pure-quality was added to the medium in amounts of 0.5, 1.0, 2.0, 3.0 and 4.0%. Thiourea was added to the medium in amounts of 0.1, 0,2, 0.3, 0.4 and 0.5%, and each medium was previously checked so as to insure normal growth of *E. coli*, because an excess of thiourea would prevent the growth of all bacteria.

Irradiation was undertaken for 2 hours at room temperature ranging between 18.0° and 18.5° C. The results of using sucrose are shown in Table IX and diagrammed in a single graph in Figure 5. As it can be seen, the survival-curves are identical. The LD 90 value for all of them was 2.3×10^4 r, which

TABLE XI
SURVIVAL RATIO OF *E. COLI* (YCI) BY γ-RAY IRRADIATION IN NUTRIENT AGAR CONTAINED THIOUREA

Average Survival Percentage

thiourea	0%	0.2%	0.3%
concentration	761 cells/ml	703 cells/ml	691 cells/ml
Dose $\times 10^3$ r			
31.9	5.0%	16.4%	14.4%
19.2	12.8	29.8	27.4
12.8	26.9	41.8	36.6
9.1	37.2	51.3	58.9
6.2	55.1	72.1	72.8

thiourea	0.1%	0.4%	0.5%
concentration	923 cells/ml	563 cells/ml	709 cells/ml
Dose $\times 10^3$ r			
24.3	16.2%	27.2%	34.2%
15.5	34.1	31.7	45.0
10.7	51.1	45.9	60.1
7.7	72.3	52.0	66.5
5.2	85.9	67.6	73.3

may indicate no effect of sucrose upon the survival of *E. coli* both during and after irradiation.

The results of using salt are shown in Table X, and also in the graph of Figure 6.

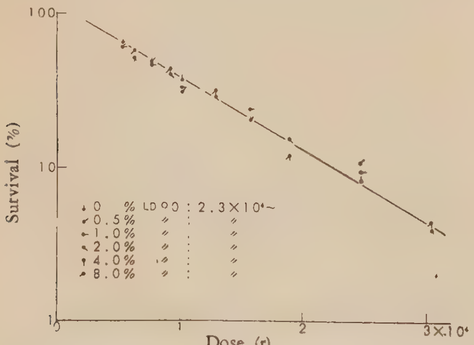

FIG. 5. Effect of Addition of Sucrose on Survival of *E. coli* (YCI).

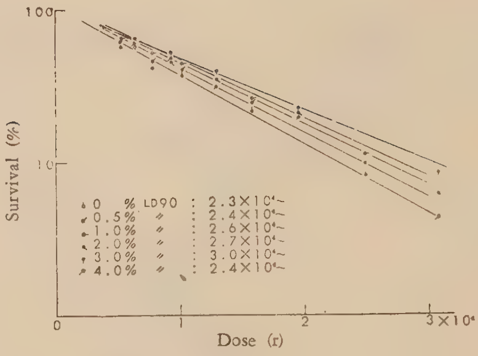

FIG. 6. Effect of Addition of NaCl on Survival of *E. coli* (YCI).

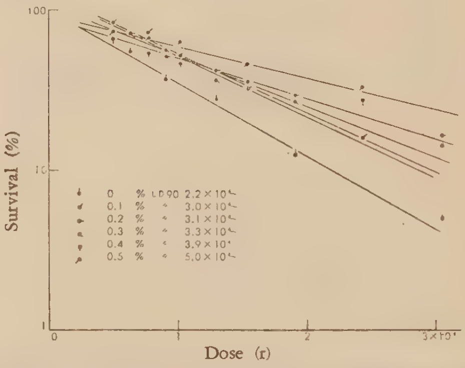

FIG. 7. Effect of Addition of Thiourea on Survival of *E. coli* (YCI).

In this case when salt is not used, the survival-curve had the lowest value the 0.5 and 4.0% curves following. With 1.0, 2.0 and 3.0% salt, the higher the concentration of salt recovered the more the survivals, LD 90 value was also larger when the concentration of salt increased, except in the case of 4.0%. However, the differences in survivals, were so small that salt seems to have very little, if any, protective effect upon the survival of *E. coli.*

The results of using thio-urea are shown in Table XI and by the single-graph in Figure 7. One can easily find the apparent effect of the addition of thio-urea both in the graph and the table of LD 90 values. Thus, when thiourea is added it is found to protect the survival of *E. coli* from gamma-ray irradiation.

In this case, it is necessary to note that both irradiation and culturing for recovery were made in the same medium. Therefore, it may be possible that protection during irradiation or restoration of activity during curing culturing may have taken place.

However, practically in Cold Sterilization, microorganisms contained in foodstuffs are irradiated and cultured in the same foodstuff (medium). Therefore, it is seems that the results obtained be of practical to a certain extent.

SUMMARY

1) Despite the bacterial concentration, the survival-curves of *E. coli* are approximately constant at the highest concentration of 10^7 cell/ml and at the lowest, 10^2 cell/ml.

2) In this case, the mass of points covers a wider range as the bacterial concentration is raised.

3) In this method, since the radiation time is shorter and there is less probability of appearance of mutants, the medium containing a concentration of 10^2 cell/ml seems to be most satisfactory.

4) A comparison of gamma-ray resistivity of each type of bacteria, using a medium of 10^2 cell/ml concentration has been made.

5) Moreover, the influence of chemical substances upon the lethal effect of gamma-ray on *E. coli* was investigated.

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Studies on the Metabolism of D-Amino Acid in Microorganisms

Part V. Oxidation of D-Glutamic Acid by Cell-free Extracts of *Aerobacter* Strain A

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Received October 22, 1957

It is confirmed by a new method for the determination of D-glutamic acid, that *Aerobacter* strain A rapidly metabolizes D-glutamic acid, while it only shows feeble metabolic activity towards L-glutamic acid when it is grown on a DL-glutamate-K_2HPO_4 medium. A specific D-glutamic oxidase is demonstrated in the cell-free extracts of *Aerobacter* strain A. This enzyme seems to be different from D-glutamic-aspartic oxidase obtained from *Aspergillus ustus* by the authors, since the former has no activity towards D-aspartic acid.

INTRODUCTION

In the previous report[1], it was described that several strains of genus *Aerobacter* isolated from sewage show good growth on D-glutamate-K_2HPO_4 medium, whereas, on L-glutamate medium only poor growth is observed. L-Glutamic acid was successfully obtained from its DL-form by the decomposition of D-glutamic acid using one of these strains. Hence, it was assumed that D-glutamic acid was oxidatively metabolized causing a rise of ammonia and α-ketoglutarate by D-glutamic oxidase.

In the present paper, the previous results are confirmed by a new method for the determination of D-glutamic acid. It is now possible to demonstrate a specific D-glutamic oxidase in a cell-free state from *Aerobacter*, which has different characteristics from fungal D-glutamic-aspartic oxidase already described by the authors[2]

METHODS AND MATERIALS

1. Media and cultural conditions. In order to investigate bacterial growth, medium A containing 1% D-glutamic acid, 0.2% K_2HPO_4, 0.02% $MgSO_4$ and 0.5% NaCl was used. Medium B which contains 1% D-glutamic acid and 0.2% K_2HPO_4 was used when large amount of cells is necessary. Both media were adjusted to pH 7.2. Cell suspension prepared from nutrient agar slant culture was inoculated to 10 ml of medium A in test tubes, and the tubes were incubated under stationary conditions at 30°C. Bacterial growth was determined by measuring turbidity at 650 mμ, using a photoelectric colorimeter. Aerobic culture condition was made by the use of Monod's shaking machine at 30°C. Bacterial cell suspension was inoculated to 10 ml of medium A in Monod's shaking tubes, and the tubes were incubated at 30°C. At appropriate times intervales, growth rates were determined by measuring turbidity using a nepherometer.

2. Materials. D-Glutamic, DL-glutamic acids, and α-ketoglutaric acid were kindly supplied by the Ajinomoto Co Ltd. D-Alanine, D-aspartic acid, and L-alanine were obtained from the California Foundation for Biochemical Research, U.S.A.. Other DL-amino acids used were products of General Biochemicals Inc., U.S.A..

3. Method for determining enzyme activity. Oxygen uptake was measured at 30°C using a Warburg respirometer. Ammonia contained in the incubation medium was distilled into N/100 H_2SO_4 by Conways' micro-diffusion unit and assayed colorimetrically after nesslerization. D-Glutamic acid was estimated manomertrically by D-glutamic-aspartic oxidase from

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[1] K. Izaki, H. Takahashi and K. Sakaguchi, This Bulletin, **19**, 233-239 (1955).

[2] S. Mizushima, K. Izaki, H. Takahashi and K. Sakaguchi, This Bulletin, **20**, 36-41 (1956).

Aspergillus ustus[3]. L-Glutamic acid was estimated manometrically by L-glutamic acid decarboxylase from *E. coli*[4], and α-ketoglutaric acid was assayed by a modification of the Friedemann and Haugens method[5].

EXPERIMENTAL RESULTS

I. Strains of bacteria which are able to grow on D-glutamate. Several strains of bacteria which show better growth on a D-glutamate medium rather than on a L-glutamate medium were isolated from soil as previously described. All of these strains were proved to belong to genus *Aerobacter* from taxonomical characteristics which are described as follows. Spore-none, gram-negative, Voges Proskauer test-positive, methyl red test-negative, good growth on citrate medium, indole formation-negative, fermentation of carbohydrates such as glucose, mannose and lactose with acid and gas, reduction of nitrate-positive, hydrogen sulfide formation-positive. Taxonomical studies of these strains will be described elsewhere.

One of the strains isolated was tentatively named *Aerobacter* strain A, which was used for this experiment. Several strains of the type culture stored in our laboratory were also tested for growth in both D- and L-glutamic acids.

These include six strains of *E. coli*, four strains of

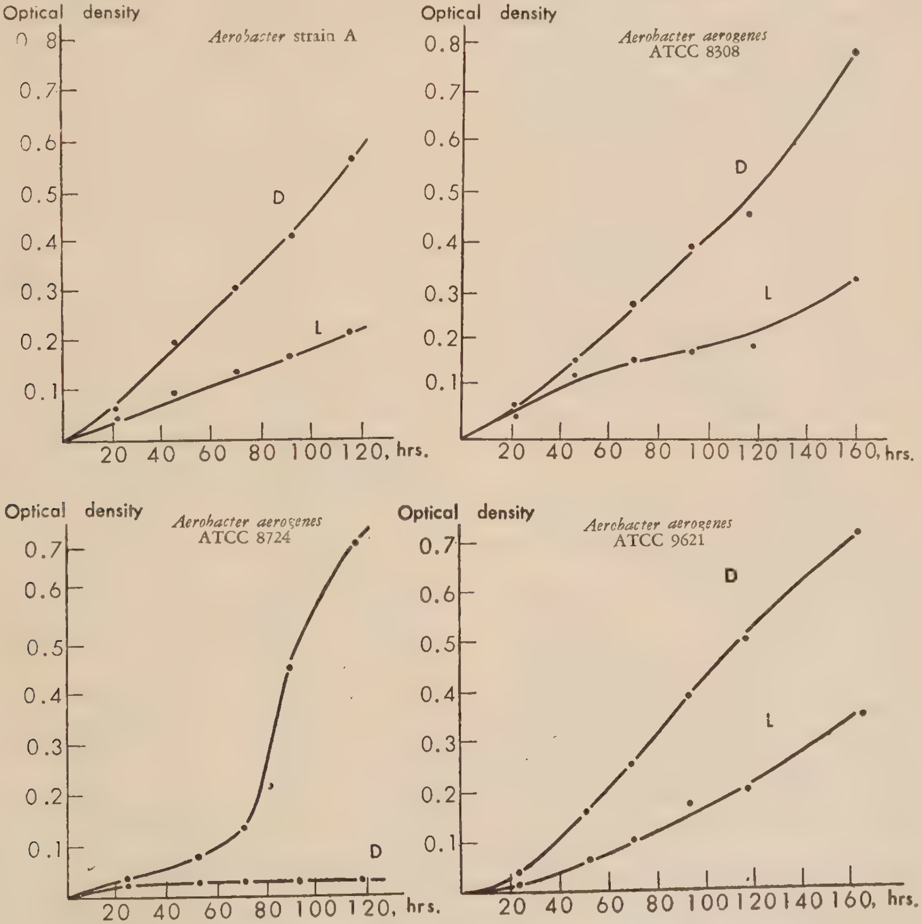

FIG. 1. Growth Curve of *Aerobacter aerogenes* ATCC 8308, 8724, 9621 and *Aerobacter* strain A.

(Glutamine-K_2HPO_4 medium, stationary condition) butylenglycol forming bacteria, and five strains of *Pseudomonas*. All strains belonging to *E. coli* and butylenglycol forming bacteria did not grow on a glutamate-K_2HPO_4 medium. Upon addition of a small amount of yeast extract to the medium, several strains showed slow growth on L-glutamate medium, however, there is no strain which grows on D-glutamate medium in the presence of yeast extracts. Five strains of *Pseudomonas* grew well on a L-glutamate medium, while no growth was observed on D-glutamate. In genus *Aerobacter*, four strains belonging to *Aerobacter aerogenes* and *Aerobacter* strain A were tested. Interestingly enough, two strains of *Aerobacter aerogenes* and *Aerobacter* strain A grew better on a D-glutamate medium than on L-glutamate medium as shown in Fig. 1. *Aerobacter aerogenes* ATCC 8729 grew on L-glutamate, whereas, it did not grow on D-glutamate medium. *Aerobacter* strain A was grown aerobically on medium A and the growth rate was measured. The result is given in Fig. 2. As shown strain A grew well on D-glutamate medium, while only poor growth was observed on L-glutamate medium.

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[3] S. Mizushima, K. Izaki, H. Takahashi and K. Sakaguchi, This Bulletin, **20**, 178–182, (1956).

[4] W.W. Umbreit and I.C. Gunsalus, *J. Biol. Chem.*, **159**, 333 (1945).

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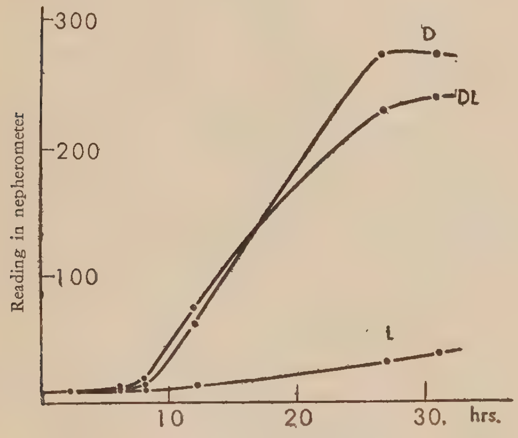

FIG. 2. Growth of *Aerobacter* strain A.

(Glutamate-K_2HPO_4 medium, aerobic condition)

II. Oxidative activities of resting cells of *Aerobacter* strain A towards amino acids at various growth-periods.

Aerobacter strain A was grown aerobically in DL-glutamic acid -K_2HPO_4 medium pH 7.2, and at various growth phases the cells were harvested and their oxidative activities towards glutamic acid, aspartic acid and alanine were estimated. At the same time, the amounts of D- and L-glutamic acids in the culture medium were determined. The results are given in Table I and Table II. Oxidative activity towards D-glutamic acid differed remarkably at different growth periods. Activity was strong at the earlier stage of culture, and decreased rapidly with the progress of incubation-time as shown in Table I. It is noteworthy that D-alanine and L-alanine are also oxidized rapidly. D-Glutamic acid disappeared within twenty-four hours, while the amount of L-glutamic acid did not decrease at all. When the culture was continued for a period over twenty-four hours, the amount of L-glutamic acid also slowly decreased. The pH of the culture medium usually gave rise to about 8.6 after forty-five hours incubation.

TABLE I

OXIDATIVE ACTIVITIES OF RESTING CELLS OF *AEROBACTER* STRAIN A TOWARDS AMINO ACIDS AT VARIOUS GROWTH PHASES

Substrate	15 hrs.	24 hrs.	45 hrs.
D-Glutamate	168 (Q_{O_2})	105 (Q_{O_2})	12 (Q_{O_2})
L-Glutamate	18	18	8
D-Alanine	24	39	11
L-Alanine	34	60	20
DL-Aspartate	8	21	6

Each vessel contains 0.5 ml cell suspension (4 mg dry weight), M/15 phosphate buffer pH 7.2 1.0 ml and 20 μM of substrate in a total volume of 1.7 ml; 0.2ml of 20% KOH is placed in the center well.

TABLE II

GLUTAMIC ACIDS CONTENT IN CULTURE MEDIUM

Glutamic acid	Initial	15 hrs.	24 hrs.	45 hrs.
D-Form	3.5 mM (per 100 ml)	3.2 mM	0 mM	0 mM
L-Form	3.6	3.9	3.9	3.1

III. The effects of pH and substrate concentration on glutamate oxidizing activity of resting

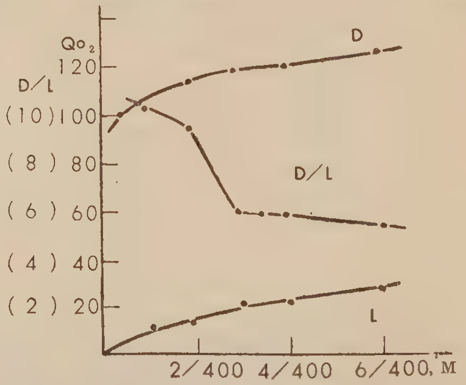

FIG. 3. The Effect of Concentration of Substrate on Glutamate Oxidizing Activity of Resting Cells.

Experimental conditions are the same as described in Table I.

cells. The effects of pH and substrate concentration on glutamate oxidizing activity of *Aerobacter* strain A were examined using resting cells obtained from twenty-four hours' culture on DL-glutamate-K_2HPO_4 medium. The relationship between oxidative activity and D-glutamate concentration is shown in Fig. 3. As seen, the rate of oxidation was almost constant at D-glutamate concentrations ranging from $\frac{1}{400}$ mol to $\frac{1}{200}$ mol. On the other hand, greater oxidative activity towards L-glutamic acid was obtained as the substrate concentration increased. Therefore, the ratio of oxidative activities towards D-glutamic acid to that towards L-glutamate, (D-G/L-G), increased when the substrate concentration was lower.

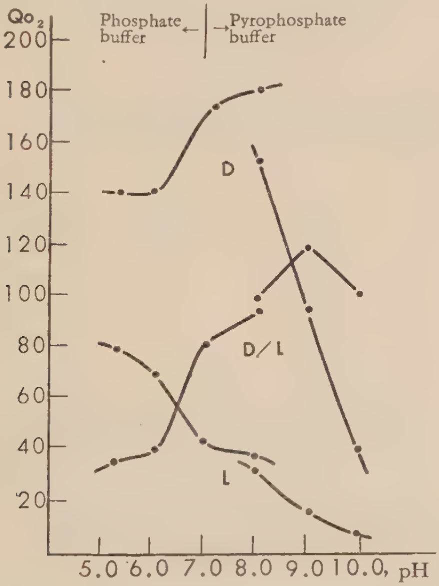

FIG. 4. The Effect of pH on Glutamate Oxidizing Activity of Resting Cells.

Experimental conditions are the same as described in Table I.

As shown in Fig. 4, the optimum pH for D-glutamate oxidation was about 8.0. On the other hand, L-glutamate was oxidized much rapidly at acid conditions, rather than at pH 8.0. The ratio D-G/L-G, therefore, increased as pH rised from 5.2 to 9.0 as shown in Fig. 4. Since pH of culture medium increases from 7.2 to 8.6 at the maximum growth-phase as described in the preceding chapter, it is favorable for D-glutamic acid oxidation, and less suitable for L-glutamate oxidation as growth proceeds.

IV. The metabolic pathway of D-glutamic acid.

There are several possibilites that D-glutamic acid is primarily metabolized by non-oxidizing enzymes such as racemase, decarboxylase, and transaminase. Since these reactions proceed under anaerobic conditions, the following experiments were carried out to examine the possibility whether these reaction actually exist. Fifty-micro moles of D-glutamic acid was incubated with cell suspensions for two hours under anaerobic conditions, and the reaction was stopped by heating the mixture, the remaining D-glutamic acid was then measured manometerically, giving a value of 50.4 micromoles. This indicates that D-glutamic acid is not metabolized under anaerobic conditions.

From these results, it is favorable that the primary step of D-glutamate metabolism seems to be an oxidation reaction.

In order to clarify the oxidative metabolism of D-glutamic acid, an experiment using whole sonicates was next attempted. As it is supposed that α-ketoglutaric acid is the primary product in D-glutamic acid oxidation, D-glutamic acid was incubated aerobically, with whole sonicates in the presence of 3 to 5× 10^{-4} M arsenite, which is known to inhibit α-keto acid oxidation in the biological system. After incubation, the remaining D-glutamic acid and the formation of α-ketoglutaric acid in the reaction mixture were estimated.

As illustrated in Table III, considerable amounts of α-ketoglutaric acid do accumulate in incubation medium of whole sonicates under these conditions. Accumulated keto acid was estimated as approximately thirty percent

TABLE III

α-KETOGLUTARIC ACID ACCUMULATED AS A PRODUCT OF D-GLUTAMIC ACID OXIDATION BY ULTRASONIC LYSATE IN THE PRESENCE OF ARSENITE

	D-G. consumed	α-Kg. formed	α-Kg/ D-G.
20 μM of D-G.	10.6 μM	0.8μM	8%
20 μM of D-G. +3×10^{-4} M arsenite	7.3	2.3	30
20 μM of D-G. +5×10^{-4} M arsenite	2.4	0.8	33

Each vessel contains 0.5 ml enzyme solution; M/15 phosphate buffer pH 7.2 1.5 ml. D-Glutamate and arsenite are added as indicated in the table, 0.2 ml of 20% KOH is placed in the center well.

The values are corrected by subtracting endogenous values. The duration of incubation is 30 minutes in the absence of arsenite, and 90 minutes in the presence of the inhibitor.

D-G: D-Glutamate
α-Kg: α-Ketoglutarate

in molar basis of the theoretical value based on the following equation:

$$\text{D-glutamic acid}+\text{O}\rightarrow\alpha\text{-ketoglutarate}+NH_3$$

Even when intact cells were incubated with D-glutamic acid in the presence of 5×10^{-5} M arsenite, twenty percent of D-glutamic acid consumed was accounted as α-ketoglutaric acid. These results suggest that D-glutamic acid is metabolized oxidatively via α-ketoglutaric acid.

V. Oxidation of D-glutamic acid by cell-free extracts of *Aerobacter* strain A. There has been some trouble to obtain a cell-free preparation capable of oxidizing D-glutamate owing to the unstable nature of the enzyme. The following method of preparation, however, gave a satisfactory results. The method was as follows. The cells were collected by centrifugation from the aerobic culture on D-glutamic acid-K_2HPO_4 medium from fifteen to sixteen hours. The cells were washed once with distilled water, and finally suspended in a phosphate buffer (pH 7.2) to give a final concentration of approximately 20 to 30 mg dry weight per ml. The cell suspension was sonicated for thirty minutes at 10 kilocycles/sec.. All the sonicates were centrifuged at 13,000×g for thirty minutes in order to remove the intact cells and the cell-debris. The supernatant solution was used as crude cell-free extracts. Oxidative activities of this preparation towards various amino acids were examined. Since enzyme activity was found to be considerably weak, the number of viable cells was counted by the plate method. Approximately 10,000 cells per ml enzyme preparation were found, and activity due to the remaining cells was calculated to less than one percent of total activity.

TABLE IV

OXIDATION OF AMINO ACIDS BY CELL-FREE EXTRACTS OF *AEROBACTER* STRAIN A.

(D-Glutamate-K_2HPO_4 medium)

Preparation number	1		2
	O_2 μM uptaken/hr.	NH_3 μM formed/hr.	O_2 μM uptaken/2 hr.
None	0.4 μM	3.2 μM	0.3 μM
D-Glutamate	2.7	7.7	2.2
D-Alanine	0.9	4.6	1.2
D-Aspartate	0.4	3.2	0.5
DL-Amino adipate	—	—	0.4
DL-Valine	0.4	—	0.8
DL-Ornithine	0.3	—	—
DL-Threonine	—	—	0.5
Glycine	—	—	0.4
L-Glutamate	0.5	3.2	0.4
L-Aspartate	0.9	15.0	—
L-Alanine	0.2	3.2	—

Each vessel contains 0.5 ml of cell-free extracts, M/15 phosphate buffer pH 7.2 1.0 ml and 0.4 ml of substrate solution. 0.2 ml of 20% KOH is placed in the center well. 80 μM of substrate as D or L in preparation 1, and 40 μM of substrate in preparation 2 are added respectively.

As shown in Table IV, only D-glutamic acid, was rapidly oxidized by this enzyme preparation. The other eleven amino acids tested were not oxidized at a measurable rate with the exception of D-alanine and L-aspartic acid. It is worthy of note here that D-aspartic acid, which is a substrate for fungus D-glutamic-aspartic oxidase, is not oxidized by the cell-free extracts. Since large amounts of ammonia is formed from L-aspartic acid and little amounts of oxygen are taken up, the crude enzyme preparation seemed to contain a pretty strong aspartase. For confirmation of this fact aspartic acid was identified by paperchromatography in the reaction mixture containing fumaric acid, ammonium chloride and cell-free extracts. D-Alanine was oxidized slowly by the cell-free extracts. The activity of D-Alanine oxidation, however, seemed to be due to an enzyme different from D-glutamic oxidase since, as shown in Table V, the ratio of two activities was considerably variable from preparation to preparation. In addition to this, the activity of D-glutamic acid oxidation decreased to

TABLE V

THE RATIO OF ENZYME ACTIVITIES TOWARDS D-GLUTAMIC ACID TO THAT TOWARDS D-ALANINE IN VARIOUS ENZYME PREPARATIONS

Preparation		D-Glutamate	D-Alanine	D-G/D-A*
1	O_2 uptaken	2.3 μM	0.5 μM	4.6 μM
	NH_3 formed	4.5	1.4	3.2
2	O_2	1.9	0.9	2.1
3	O_2	1.7	1.6	1.1
	NH_3	3.1	3.1	1.0
3′	O_2	0.8	1.6	0.5
	NH_3	1.9	3.7	0.5

Each vessel contains 0.5 ml of cell-free extracts, M/15 phosphate buffer pH 7.2 1.0 ml, and 0.2 ml of 20% KOH is placed in the center well; 0.4 ml of substrate solution (80 μM) is added as indicated in the table.

The values are corrected by subtracting endogenous values. The duration of incubation is 120 minutes.

The enzyme preparation 3′ is obtained by treating preparation 3 at 45°C for 30 minutes.

* The ratio of oxidative activity towards D-glutamic acid to that towards D-alanine.

almost half of the original level after heat-treatment (45°C, for thirty minutes), while there was no effect on the activity of D-alanine oxidation. Furthermore, cell-free extracts from the organism grown on casein hydrolyzate-pepton medium, oxidized D-alanine rapidly, while it had no activities towards D-glutamic and D-aspartic acids as shown in Table VI. The author was unable to detect any activity of transaminase reaction between D-alanine and D-glutamic acid. It was therefore concluded from these results, that D-glutamic acid and D-alanine are activated by separate enzymes, and D-glutamic acid is oxidized by a specific oxidase.

TABLE VI

OXIDATION OF AMINO ACIDS BY RESTING CELLS AND CELL-FREE EXTRACTS

(Pepton-caseinhydrolyzate-medium)

Experiment No.	1	2
Preparation	Resting cells	Cell-free extracts
None	0.4μM O_2 uptaken/hr.	0.0μM uptaken/hr.
D-Glutamate	0.3	0.0
D-Alanine	7.0	2.0
D-Aspartate	0.6	0.0
DL-Valine	0.8	0.2
DL-Methionine	—	0.7
DL-Ornithine	—	0.3
L-Glutamate	—	0.0
L-Alanine	—	1.0

Each vessel contains enzyme solution or resting cell suspension (2.5mg dry weight) 1.0 ml, M/15 phosphate buffer pH 7.2 1.0 ml, 0.2 ml of 20% KOH is placed in the center well.

20μM of substrate in experiment 1 and 80μM of substrate in experiment 2 are added, respectively.

VI. Stoichiometry of D-glutamic acid oxidation. Eighty-micro moles of D-glutamic acid was incubated with a crude extract, and at an appropriate time, the reaction was stopped by tipping 50 percent trichloroacetic acid, and the amounts of oxygen uptake ammonia and α-ketoglutaric acid formations were assayed. Judging from the result presented in Table VII, the reaction proceeds according to the following equation.

$$\text{D-glutamate} + \text{O} \rightarrow \alpha\text{-ketoglutarate} + \text{NH}_3$$

TABLE VII

STOICHIOMETRY OF D-GLUTAMIC ACID OXIDATION

O_2 uptaken	α-Ketoglutarate formed	NH_3 formed
2.3μM	3.5μM	4.5 μM
1.7	3.0	3.2

Experimental conditions are the same as described in Table IV.

VII. Properties of D-glutamic oxidase.

1. Optimum pH: The effect of pH on enzyme activity is shown in Fig. 5. The pH optimum was estimated to be around about 7.5*.

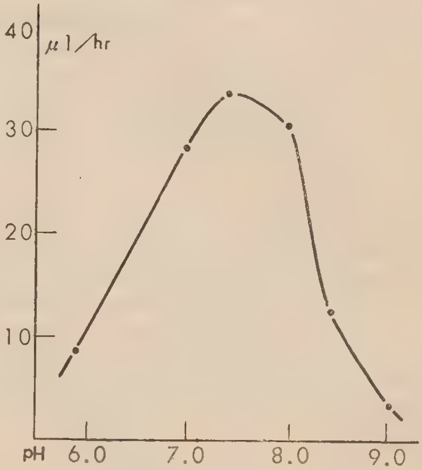

FIG. 5. The Effect of pH on D-Glutamate Oxidizing Activity of Cell-free Extracts.

TABLE VIII

EFFECTS OF INHIBITORS ON ENZYME ACTIVITY

Inhibitor	Concentration	Inhibition %
Para-chloromercuri-benzoate	10^{-3} M	100
	10^{-4} M	100
	10^{-5} M	0
KCN	10^{-2} M	100
	10^{-3} M	20–50
NaN_3	10^{-3} M	50
NH_2OH	10^{-3} M	30
Versene	10^{-3} M	0
Semicarbazide	10^{-3} M	0
8-Hydroxyquinoline	10^{-3} M	0
Monoiodoacetate	10^{-3} M	0
$\alpha\alpha'$-Dipyridil	10^{-3} M	0
Benzoate	10^{-3} M	0

Enzyme activity is determined by a Warburg respirometer. Each vessel contains 0.5 ml of cell-free extracts, M/15 phosphate buffer pH 7.2 1.0 ml and 0.2 ml of inhibitor solution in the main compartment, and 80μM of D-glutamate in the side arm. 0.2 ml of 20% KOH is placed in the center well.

2. The effect of inhibitor: Effects of various inhibitors on D-glutamic oxidase are shown in Table VIII. Para-chloromercuri-benzoate, potassium cyanide inhibited enzyme activity at such low concentrations

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* Borate phosphate buffer was used.

as 10^{-3} M, but chelating agents such as versene, 8-hydroxyquinoline, αα′-dipyridil showed no effects at the concentration of 10^{-3} M. It is noteworthy that both sodium azide and hydroxylamine inhibit enzyme activity at the concentration of 10^{-3} M.

3. Cofactor: Enzyme activity decreased about one-third as much as the crude extract after dialysis against pH 7.2 phosphate buffer at 2°C for about forty hours. Enzyme activity was not restored by the addition of TPN, DPN, FAD, FMN* or boiled crude extract. Therefore, the co-factor seems to be strongly bound to apoenzyme.

4. Electron acceptor: When D-alanine, or D-glutamic acid was used as the substrate, dialyzed enzyme preparation rapidly reduced 2,6-dichlorophenol indophenol under anaerobic conditions as shown in Table IX.

TABLE IX

REDUCTION OF 2,6-DICHLOROPHENOLINDOPHENOL

Substrate	Time required for reduction
D-Glutamic acid	5–6 minutes.
D-Alanine	11–12
D-Aspartic acid	>120
None	>120

Each thunberg tube contains enzyme solution (dialyzed) 0.5 ml, M/15 phosphate buffer pH 7.2 4.0 ml, and M/1000 dye solution 1.0ml and 0.5 ml of M/5 substrate solution.

Incubation at 30°C under anaerobic condition.

On the other hand, the reductions of methylene blue, thionine were very slow.

DISCUSSION

Interestingly enough, several strains of genus *Aerobacter* can preferantially metabolize D-glutamic acid when they are grown on a medium containing DL-glutamic acid as the sole source of carbon and nitrogen. The reason why these strains metabolize unnatural amino acid better than natural amino acid still remains unknown.

One possibility is that of the permeability effect, that is, D-glutamic acid can readily penetrate through the cell-wall, while L-glutamic acid cannot in case of these strains. The observation that L-glutamic acid is oxidized faster under acid condition than under neutral enviroments seems to support the above hypothesis.

The second possibility is that only D-glutamic acid is available as an energy source in this organism whereas, this is not the case of the L-isomer owing to lackness of enzyme activity acting on the L-isomer. The third reason is that D-glutamic acid plays a somewhat important physiological role in this organism. It is of interest to note that the capsular material of *Bacillus subtilis* does contain D-polyglutamic acid as constituents. It has been already established that a single oxidase can activate both D-glutamic and D-aspartic acids in *Aspergillus ustus*. It is thus evident from the results of this report, that oxidase from *Aerobacter* strain A can solely oxidize D-glutamic acid. It was also observed that the oxidase obtained from *Aerobacter* strain A is remarkably labile compared to D-glutamic-aspartic oxidase in *Aspergillus ustus*. Therefore, in spite of many similarities in their properties, D-glutamic oxidase in genus *Aerobacter* is different from D-glutamic-aspartic oxidase in *Aspergillus*, both in their specificity and stability features.

Acknowledgement. The author wishes to express his sincere thanks to Prof. K. Sakaguchi for his constant guidance throughout the course of this work. Indebts are also due to the Ajinomoto Co. Ltd. for generously suplying the D-glutamic acid used here.

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* TPN: Triphosphopyridine nucleotide
DPN: Diphosphopyridine nucleotide
FAD: Flavin-adenine-dinucleotide
FMN: Flavin mono nucleotide

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 2, p. 85~91, 1958]

Studies on Browning Reactions between Sugars and Amino Compounds[1]

Part III. Identification of Red Pigments and Furfural Produced from Aromatic Amine-N-Xylosides

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Received October 24, 1957

Aromatic amine-N-xylosides were found to produce both melanoidins and red pigments in methanol solution acidified with hydrogen chloride at 25°. From N-D-xylosyl-*p*-aminobenzoic acid, 1-*p*-carboxyphenylimino-5-*p*-carboxyphenylamino-2-hydroxypenta-2,4-diene hydrochloride, and from N-D-xylosylaniline, 1-phenylimino-5-phenylamino-2-hydroxypenta-2,4-diene hydrochloride were isolated and identified respectively. And further, furfural formed from N-D-xylosyl-PABA or N-D-xylosylaniline under the same condition was identified as 2,4-dinitrophenylhydrazone of which anti-form and syn-form were clearly separated by adsorption chromatography with alumina.

Rosen, Johnson and Pigman[2] studied the browning of N-D-glucosylaniline in methanol solution acidified with hydrogen chloride, and they discussed the role of 5-hydroxymethylfurfural (HMF) in browing, but could not obtain any proof. By many investigators the importance of furfural or HMF in the browning mechanism has been shown chiefly on experimental basis by paperchromatography[3~10].

In part II of this series[1], it has been concluded that N-glycosyl-*p*-aminobenzoic acid (N-glycosyl-PABA) is unstable in the acidic condition that causes browning. At 25°, N-D-xylosyl-PABA in methanol suspension acidified with hydrogen chloride gradually dissolves with the formation of a reddish-black color of which intensity successively increases after complete solution. The spectrum of this colored solution (Fig. 1) indicates the co-existence of a red pigment and melanoidin. In the hope of elucidation of the

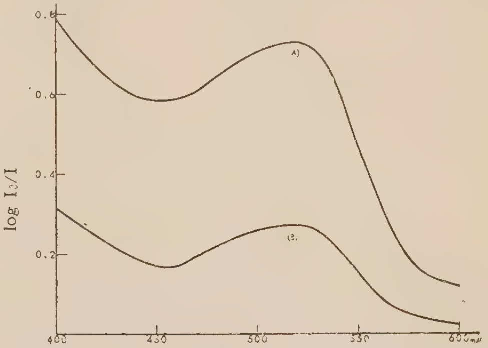

FIG. 1. Pigment Formation of N-D-Xylosyl-PABA.

(A) 0.2M solution of N-D-xylosyl-PABA in methanolic hydrogen chloride (0.2 N) stood at 30° for twenty hours and diluted to 0.001 M.

(B) 0.1 M solution of N-D-xylosyl-PABA in methanolic hydrogen chloride (0.1 N) was stood at 25° for twenty hours and diluted to 0.001 M.

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mechanism of melanoidin formation between sugars and amino compounds, this red pigment is investigated in the present paper.

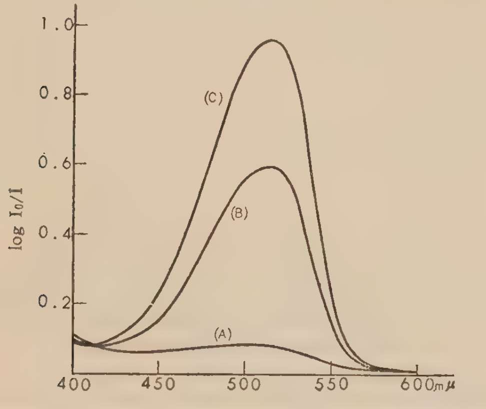

FIG. 2. Pigment Formation of N-D-Xylosylaniline.

(A) 0.5 M solution of N-D-xylosylaniline in methanolic hydrogen chloride (0.5 N) was stood at 25° for twenty hours, and (B) barium carbonate was added and allowed to stand for three hours and filtered, and (C), to the filtrate methanolic hydrogen chloride (1 N) was added. All of them were diluted to 0.0002 M.

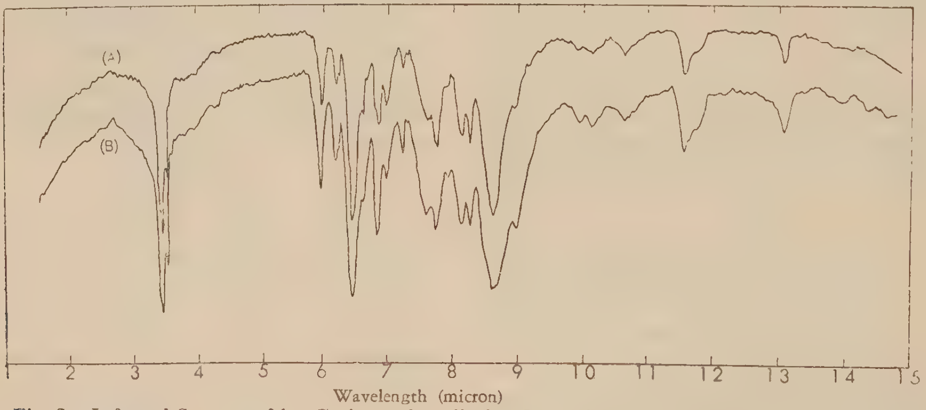

Fig. 3. Infrared Spectra of 1-*p*-Carboxyphenylimino-5-*p*-carboxyphenylamino-2-hydroxypenta-2,4-diene Hydrochloride Monohydrate in Nujol Mull.

(A) synthesized from furfural and PABA.

(B) isolated from the colored reaction mixture of N-D-xylosyl-PABA.

It has been identified that the red pigment ($C_{19}H_{19}N_2O_6Cl$, m.p. 221°) which has been isolated in crystalline powder from acidified (hydrogen chloride) methanol solution of N-D-xylosyl-PABA at 25° for twenty hours, is the same with the substance obtained from furfural and PABA in methanolic hydrogen chloride by both mixed melting point and infrared spectra (Fig. 3). Furthermore, N-D-xylosylaniline also forms a reddish black color under the same condition. In this case, the addition of barium carbonate after twenty hours at 25° increases formation of the red pigment strikingly (Fig. 2). This red pigment ($C_{17}H_{19}N_2O_2Cl$, m.p. 171°) which has been isolated in crystalline from the reaction mixture of N-D-xylosylaniline is the same with the compound formed from furfural, aniline and hydrochloric acid as in the case of N-D-xylosyl-PABA (Fig. 4). Since Stenhouse[11] first reported on the reaction of furfural with aniline and aniline hydrochloride, many investigators have studied on the chemical structure of this dye[12~15]. From their results, its most reliable structure is 1-phenylimino-5-phenylamino-2-hydroxypenta-2,4-diene hydrochloride (I). As shown in Figs. 3 & 4, the resemblance between the infrared spectrum of the red dye formed

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[11] J. Stenhouse, *Ann.*, **156**, 197 (1870).

[12] T.H. Zincke and G. Muehlhausen, *Ber.*, **38**, 3824 (1905).

[13] G. Williams and C.L. Wilson, *J. Chem. Soc.*, **1942**, 506.

[14] J.C. McGowan, *ibid.*, **1949**, 777.

[15] W.M. Forey, Jr., G.E. Sanford and H. Mckennis, Jr., *J. Am. Chem. Soc.*, **74**, 5489 (1952).

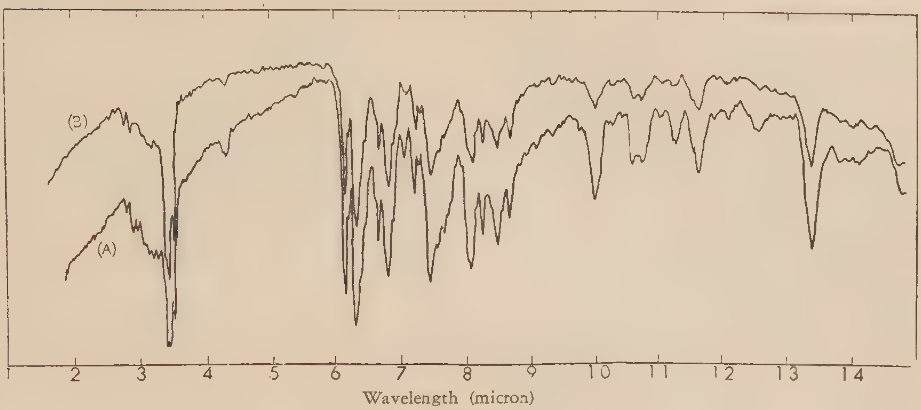
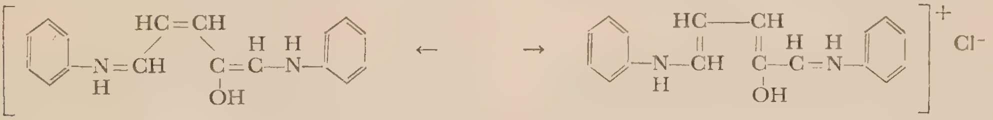
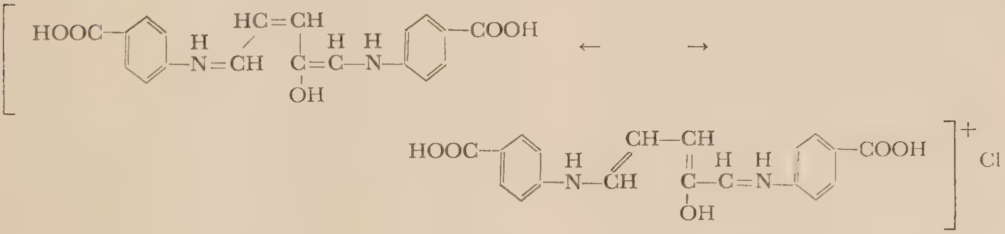


FIG. 4. Infrared Spectra of 1-Phenylimino-5-phenyl-amino-2-hydroxypenta-2,4-diene Hydrochloride Monohydrate in Nujol Mull.

(A) synthesized from furfural and aniline.
(B) isolated from the colored reaction mixture of N-D-xylosylaniline.



(I)



(II)

from aniline and that of the red pigment formed from PABA shows that the latter is quite an analogous reaction product with the former. Therefore, it is confirmed that the structure of the latter is 1-*p*-carboxyphenylimino-5-*p*-carboxyphenylamino-2-hydroxypenta-2,4-diene hydrochloride (II). As described above, in the case of N-D-xylosylaniline, the effect of barium carbonate is probably based on the mechanism in which barium carbonate neutralizes hydrogen chloride partially, free aniline then reacting with furfural to form anil of furfural which reacts with aniline hydrochloride to produce the red dye. The theoretical yield in molar ratio of pigment from N-D-xylosylaniline or N-D-xylosyl-PABA was approximately 7%.

The presence of furfural in the reaction mixture has been proved as its 2,4-dinitrophenylhydrazone by adsorption chromatography with silicic acid16). The yield of furfural from N-D-xylosyl-PABA (0.4 M) in methanolic hydrogen chloride (0.4 N) at 25° for twenty hours was 20%. And also from N-D-xylosylaniline (0.5 M) in methanolic hydrogen chloride

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16) F.A.H. Rice and L. Fishbein, *ibid.*, **78**, 1005 (1956).

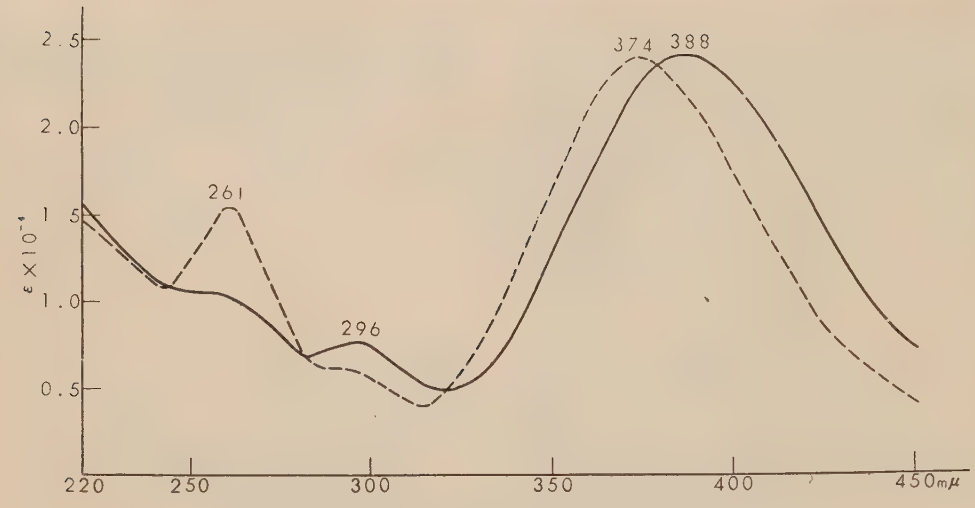

FIG. 5. Furfural 2,4-Dinitrophenylhydrazone (cf. ref. 18).

---- syn-form (yellow crystal). anti-form (red crystal).

(0.5 N) at 25° for only two hours, furfural was obtained in a yield of 14%. It is now apparent that furfural is the main-product of aromatic amine-N-xylosides under acidic (hydrogen chloride) condition.

Furfural 2,4-dinitrophenylhydrazone contains both the anti-form (red crystal, m.p. 225°) and syn-form (yellow crystal, m.p. 203°) which must be separated for conclusive identification[17~19]. In ordinary cases, a red crystal which has less solubility is obtained by recrystallization. But in certain conditions, for example, heating in acidified alcoholic solution, the ratio of yellow crystal increases resulting in difficulty to obtain red crystal only by recrystallization. This difficulty has been solved by adsorption chromatography with alumina (Brockmann) because two epimers are separated clearly into two bands of yellow and orange (red crystal) in elution with a solvent system of benzene-petroleum ether (1 : 1 by vol.). This method shall be able to serve effectively for identification of a small amount of furfural.

Pacsu and Hiller[20], observed that D-glucose solution in 50% sulfuric acid for twenty-two hours at room temperature shows strong absorption at wave length 284 mμ, in spite of the absence of HMF, although sucrose in 1 N sulfuric acid under boiling for three minutes gives a strong positive test for HMF. The author has observed that D-xylose (0.5 M) in methanolic hydrogen chloride (0.5 N) at 25° for twenty-four hours dissolves to form O-methyl-D-xyloside and gives a negative furfural test (by adding excess aniline directly), although D-xylose (1 M) in methanolic hydrogen chloride (1 N) under refluxing for one hour gives a very slight positive furfural test. Namely, sugar itself does not form furfurals in aqueous or anhydrous mineral acidic conditions at room temperature at least for forty-eight hours so far as the author's experiments are concerned. On the other hand, 1-deoxy-1-*p*-toluino-D-fructose, a crystalline compound of Amadori rearrangement product, is rather stable under the same condition as described in a previous paper of this series[1]. Hence, this furfural formation is a characteristic

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[17] H. Bredereck, *Ber.*, **65**, 1833 (1932).
[18] L.I. Braddock, et al., *Anal. Chem.*, **25**, 301 (1953).
[19] A. Wahhab, *J. Am. Chem. Soc.*, **70**, 3580 (1948).
[20] E. Pacsu and L.A. Hiller, Jr., *ibid.*, **70**, 523 (1948).

reaction of aromatic amine-N-glycosides[21].

EXPERIMENTAL

All melting points were uncorrected. 2,4-Dinitrophenylhydrazine solution was prepared by Shriner's method[22]. All spectrophotometric measurements were carried out with a Hitachi Photo-Electric Spectrophotometer in methanol solution. Infrared spectra were measured by a Kōken DS-301 Infrared Spectrophotometer.

Preparation of N-D-Xylosyl-PABA (Based on Inouye[23]). Five grams of finely powdered D-xylose, 4.8 g of PABA and 0.1 g of ammonium chloride suspended in 60 ml of 95% ethanol were refluxed in a water bath with stirring for ten minutes, and stood in the refrigerator for overnight. The crystal was filtered with suction, washed with absolute ethanol and ether, and dried. The yield was 7.7 g of fine needles (86%, m.p. 173°, dec.). Recrystallization was attained by dissolving in a large quantity of hot methanol and standing the solution in a refrigerator for three days. The yield was 3.9 g of needles, m.p. 174°, dec.). *Anal.* Calcd. for $C_{12}H_{15}NO_6$: C, 53.53; H, 5.62; N, 5.20%. Found: C, 53.70; H, 5.48; N, 5.22%.

Preparation of N-D-Xylosylaniline[24]. Five grams of finely powdered D-xylose, 4.0 g of aniline, and 1.5 ml of water were heated in a water bath at 80–85° with shaking the flask by hand at intervals, for fifteen minutes. After heating, 5 ml of absolute ethanol was added. When crystallization occurred, 20 ml of ether was added and it was stood in the refrigerator for overnight. The crystal was filtered with suction, washed with ether and dried. An amount of 3.8 g of needles was obtained (m.p. 144–145°, yield, 51%). Recrystallization from absolute ethanol gave 1.3 g, m.p. 146–147°. *Anal.* Calcd. for $C_{11}H_{15}NO_4$: C, 58.65; H, 6.71; N, 6.22%. Found: C, 58.43; H, 6.81; N, 6.25%.

Preparation of 1-Phenylimino-5-phenlamino-2-hydroxypenta-2,4-diene Hydrochloride[13]. Ten ml of aniline and 4.6 ml of freshly distilled furfural were dissolved in 50 ml of 99% ethanol and, to this solution 4.6 ml of conc. hydrochloric acid mixed with 6 ml of 99% ethanol were added (aniline, furfural and hydrogen chloride, 2 : 1 : 1, in molar ratio). After one hour, the crystalline dye was filtered with suction, washed with a mixture of absolute ethanol and ether (1 : 2 by vol.), and dried in a desiccator over calcium chloride. An amount of 15.5 g of purple scaly crystal was obtained (yield, 93%, m.p. 171°). Recrystallization from methanol gave violet prisms, 7.8 g, m.p. 172°. *Anal.* Calcd. for $C_{17}H_{17}N_2OCl \cdot H_2O$: C, 64.04; H, 6.01; N, 8.79; Cl, 11.12%. Found: C, 64.14; H, 6.18; N, 8.75; Cl, 10.68%. The absorption spectrum: λ_{max}, 247 mμ (ε, 8.11×10^3), λ_{max}, 283 mμ (ε, 4.42×10^3), λ_{max}, 518 mμ (ε, 4.18×10^4).

This crystal sublimed at heating, and at 100° under reduced pressure (2 mm/Hg, for nine hours), lost one mole of water of crystallization without any deformation of crystal. Determination of the water of crystallization: Calcd. for $C_{17}H_{17}N_2OCl \cdot H_2O$, 5.65%. Found, 5.84%. The anhydrous crystal, m.p. 171°, did not sublime, and upon admixture with the hydrated crystal melted at 167°. *Anal.* Calcd. for $C_{17}H_{17}N_2OCl$: C, 67.88; H, 5.70; N, 9.31; Cl, 11.79%. Found: C, 67.58; H, 5.55; N, 9.22; Cl, 11.87%. The absorption spectrum (220–600 mμ) was quite the same with the crystal monohydrate.

Preparation of 1-*p*-Carboxyphenylimino-5-*p*-carboxyphenlamino-2-hydroxypenta-2,4-diene Hydrochloride. A portion of 1.6 g of PABA and 0.56 g of freshly distilled furfural were dissolved in 57 ml of methanol, and to this solution 0.37 ml of conc. hydrochloric acid was added (formed a solution of 0.2 M of PABA and 0.1 M of each furfural and hydrogen chloride). This mixture was stood in the dark for twenty hours. The precipitated violet crystalline powder was filtered, washed with methanol and ether, and dried. The yield was 0.93 g (39%, m.p. 229°, (dec.)). Recrystallization was impossible according to the very little solubility and sensitivity to light of this substance. The diluted methanol solution faded immediately, subsequently, spectrophotometric measurement for this pigment itself resulted in fail. *Anal.* Calcd. for $C_{19}H_{17}N_2O_5Cl \cdot H_2O$: C, 56.09; H, 4.71; N, 6.89; Cl, 8.72%. Found: C, 55.58; H, 4.58; N, 7.08; Cl, 8.31%.

Isolation of 1-*p*-Carboxyphenylimino-5-*p*-carboxyphenlamino-2-hydroxypenta-2,4-diene Hydrochloride from Colored Reaction Mixture of N-D-Xylosyl-PABA.

(a) A 538 mg sample of N-D-xylosyl-PABA suspended in 5 ml of methanolic hydrogen chloride (0.4 N) was stood at 25° for twenty hours. The resulting colored solution was filtered and the filtrate was allowed to stand in the refrigerator for five days. The precipitated crystalline powder was then filtered, washed with methanol and ether, and dried; 20 mg was obtained, m.p. 221° (dec.).

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[21] The formation of HMF from aromatic amine-N-hexoside and the formation mechanism of the melanoidin which is formed together with a red pigment in the reaction mixture as described above (Figs. 1 & 2) will be examined in a coming paper.

[22] R.L. Shriner and R.C. Fuson, "The Systematic Identification of Organic Compounds", p. 171 (1948).

[23] Y. Inouye, K. Onodera and S. Kitaoka, *J. Agr. Chem. Soc. Japan*, **25**, 59 (1951–52).

[24] F. Weygand, *Ber.*, **72**, 1663 (1939).

(b) A 807 mg sample of N-D-xylosyl-PABA suspended in 15 ml of methanolic hydrogen chloride (0.2 N) was stood at 30° for twenty hours. The reaction mixture was filtered and the filtrate was concentrated to 5 ml in a tared conical tube blown by a stream of air with suction, and allowed to stand in the refrigerator for three weeks. The precipitate was filtered, washed with methanol and ether, and dried. A purple crystalline powder, 89 mg, was obtained (yield in molar ratio, 7.3%, m.p. 221° (dec.)). Both crystals of (a) and (b) melted at 222–223° (dec.) upon admixture with the compound synthesized from furfural and PABA, m.p. 229°, and both infrared spectra were equal (Fig. 3).

Isolation of 1-Phenylimino-5-phenylamino-2-hydroxypenta-2, 4-diene Hydrochloride from Colored Reaction Mixture of N-D-Xylosylaniline. Two grams of N-D-xylosylaniline in 17.6 ml of methanolic hydrogen chloride (0.5 N) was stood at 25° for twenty hours. The solution soon turned orange-red and then reddish-brown. To this resulting solution 1.0 g of barium carbonate was added and it was allowed to stand with stirring at intervals for three hours to cause the increase of its red color, remarkably. This solution was filtered, and to the filtrate 4 ml of methanolic hydrogen chloride (1 N) was added (Fig. 2). After thirty minutes, 20 ml of absolute ether was added to it, and again after thirty minutes, the resulting precipitate was filtered off, and the filtrate concentrated to 10 ml in volume under reduced pressure in a stream of nitrogen, and allowed to stand in the refrigerator. After two days, the crystalline precipitate was filtered, washed with a mixture of methanol and ether (1 : 2 by vol.), and dried. Crystalline, 190 mg, was obtained (m.p. 171°, yield, 6.7%). Recrystallization from methanol gave 80 mg of purple platelets, m.p. 171°. Melting point upon mixing with the compound synthesized from furfural and aniline was unchanged. Furthermore their infrared spectra were equal (Fig. 4).

Isolation of Furfural 2,4-Dinitrophenylhydrazone (Chromatographic Technique A, Based on Rice[16)]).

Adsorbent: Silicic acid (Mallinckrodt)—Hyflo-supercel (Johnsmanville—Wakō), 2 : 1 by weight.

Sample: A mixture of 2,4-dinitrophenylhydrazones prepared from the reaction mixture of N-D-xylosyl-PABA or -aniline in acidified methanol.

Chromatography: The adsorbent suspended in a mixture of benzene and petroleum ether (b.p. 60–80°) (1 : 1 by vol.) was packed in a column (diameter, 20 to 30 mm), and the sample dissolved in the same solvent system was charged. The column was washed with 100 ml of the same solvent, and then developed with benzene containing 0.5 ml of *t*-butyl alcohol per liter. An orange band of furfural 2,4-dinitrophenylhydrazone was first eluted as the main band and it was then dried up[25)].

Separation of Anti-form and Syn-form of Furfural 2,4-Dinitrophenylhydrazones (Chromatographic Technique B).

Adsorbent: Alumina (Brockmann).

Sample: (a) Furfural 2,4-dinitrophenylhydrazone separated by chromatographic technique A. (b) To furfural in 95% ethanol added 2,4-dinitrophenylhydrazine solution[22)], and the resulting precipitate filtered, washed with 50% ethanol, and dried.

Chromatography: The alumina suspended in the same benzene-petroleum ether system was packed in a column of 12×100 mm or 20×150 mm, and the sample dissolved in the same solvent was charged. After charging, development with the same solvent was continued until the yellow band was completely separated from the orange band and then eluted out from the column. Then the orange band which was situated about half-way up of the column was eluted with benzene only. Drying up the solvent gave yellow needles (m.p. 203°) from the yellow band, and from the orange band crimson prisms (m.p. 225°) were obtained.

Identification of Furfural Formed from N-D-Xylosyl-PABA in Methanolic Hydrogen Chloride. A portion of 1.2 g of N-D-xylosyl-PABA suspended in 11.2 ml of methanolic hydrogen chloride (0.4 N) was stood at 25° for twenty hours. The resulting colored solution was filtered, and to the filtrate 2,4-dinitrophenylhydrazine (0.4 g) solution[22)] was added. After thirty minutes, the precipitate was filtered, washed with 50% ethanol, and dried (yield, 320 mg). This was dissolved in the same solvent system of benzene-petroleum ether and filtered. The filtrate was charged according to chromatographic technique A, and the

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25) The fact that the band of furfural derivative is the lowest indicates that this sample does not contain any formaldehyde, acetaldehyde and crotonaldehyde of which bands must be lower than furfural, according to Rice (ref. 16).

first eluated main band was collected and dried up (yield, 240 mg). This was recrystallized from a mixture of ethanol and ethylacetate (1 : 1 by vol.). Only a red crystal was obtained (yield, 75 mg, m.p. 225). Upon admixture with the red crystal (m.p. 225) of furfural 2,4-dinitrophenylhydrazone separated by chromatographic technique B, the melting point was unchanged. The theoretical yield of the mixture of anti- and syn-form is accounted as 20%.

Identification of Furfural Formed from N-D-Xylosylaniline in Methanolic Hydrogen Chloride. A portion of 0.5 g of N-D-xylosylaniline in 4.4 ml of methanolic hydrogen chloride (0.5 N) was stood at 25° for two hours. To the resulting colored solution, 2,4-dinitrophenylhydrazine solution was added. After thirty minutes the precipitate was filtered, washed with 50% ethanol and dried (yield, 155 mg). By chromatographic technique A, the band of furfural 2,4-dinitrophenylhydrazone was collected (yield, 85 mg, 14%). Then chromatographic technique B was applied in order to obtain only a yellow crystal. The melting point of this yellow crystal (m.p. 203) did not depress upon admixture with the yellow crystal (m.p. 203) obtained from furfural.

Acknowledgement. The author wishes to express his sincere gratitude to Prof. Yusuke Sumiki for his constant guidance throughout the course of this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 2, p. 91~97, 1958]

Germination Promotant for Plants Seed, produced by Microorganisms

Part II. Germination Promotant for Rice Plant Seeds, produced by *Streptomyces* sp. S-580. Culture Condition and Extraction of the Active Crude Syrup (A.C.S.)*

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Received November 5, 1957

The culture filtrate of the *Streptomyces* sp. S–580 showed strong activity in promoting the germination of rice plant seeds. The culture conditions of the strain and fractionation of the active principles from the culture filtrate were investigated. The active crude syrup (A.C.S.) obtained is effective at the dilution of $10^{-6}\sim10^{-8}$ for the germination of rice plant seeds, $10^{-5}\sim10^{-7}$ for barley and oats seeds.

INTRODUCTION

In the previous paper1), it has been reported by Y. Koaze, H. Sakai and K. Arima that the culture filtrates and/or mycelium extracts of some microorganisms had strong promoting effects on the germination of some plants seed in the laboratory test. Especially, the culture filtrate of *Streptomyces* sp. S-580 showed remarkable strong activity in promoting the germination of rice plant seeds as shown in Fig. 1. Therefore, the author attempted to isolate the active factor from this culture filtrate and succeeded in obtaining it. In this paper, the culture conditions of this strain and pro-

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* Presented at the Meeting of the Agricultural Chemical Society of Japan held in Tokyo, October 26, 1957.

1) Y. Koaze, H. Sakai and K. Arima, *J. Agr. Chem. Soc. Japan*, **31**, 338 (1957).

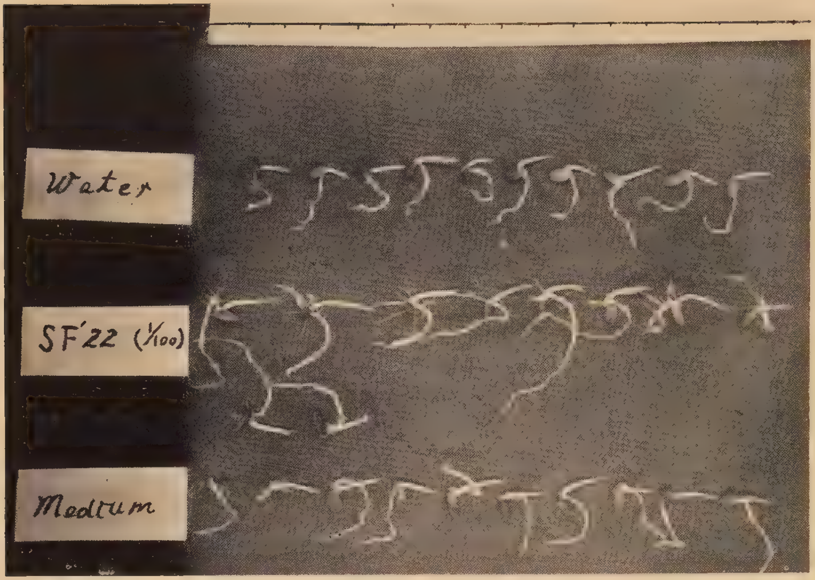

FIG. 1. Effect of the Culture Broth Filtrate of *Streptomyces* of S-580 on the Germination of the Rice Plant Seed.

Ten days after start of the experiment under the dark condition incubated at 18°C. Seeds in the middle row were treated with the cultural filtrate of *St.* sp. S-580 diluted to one hundredth. Seeds in the lower row were treated with the culture medium of this streptomyces in the same dilution and seeds in the upper row with distilled water, both are for controls.

cedures of fractionation of the active principles from the culture filtrate were investigated. Furthermore, the active crude syrup (A.C.S.) which was obtained by extracting the culture filtrate of *Streptomyces* sp. S-580 with ethyl acetate, could strongly promote the germination of rice plant seeds in the concentration range of 1 ml $\times 10^{-6} \sim 10^{-8}$, and of barley and oats seeds in the concentration range of 1 ml $\times 10^{-5} \sim 10^{-7}$.

EXPERIMENTAL AND RESULTS

1) Rice plant seed used. Fujisaka-No. 5 harvested in 1955 and 1956, and Norin-No. 1 harvested in 1955 were used in the experiments. In each experiment, the rice plant seeds which were harvested in the same year when the experiment were carried out, were used.

2) Conditions on the germination of rice plant seeds. Conditions on the germination of rice plant seeds were the same as reported in the previous paper1). However, seeds were disinfected for ten to twenty minutes and twenty to thirty seeds were laid in a germinating dish.

3) Determination of activity of the test solution in promoting the germination of rice plant seeds. Ten days after start of experiment, the length of coleoptile (or prophyll) and seminal root were measured and at the same time the formation of prophyll, crown root and root hair were observed on the each seed. Furthermore five days after start of experiment, the percentage of the germinated seeds with coleoptile elongated more than 0.5 cm and seminal root elongated more than 1 cm, against whole seeds, were measured. Results of above mentioned observations on the germination of rice plant seeds treated with a test solution, were synthesized and so, activity of a test solution in promoting the germination of rice plant seeds was determined in comparison with control.

4) Preparation of the test solution. Under the aseptic condition, the mycelium was removed from the culture broth according to the procedure reported in the previous paper1), and either the culture filtrates or the fractions extracted from the culture filtrates were diluted with sterilized distilled water and used as the test solution for determination of activity in promoting the germination of rice plant seeds.

TABLE I
MICROBIOLOGICAL CHARACTERISTICS OF *STREPTOMYCES* sp. S-580

Observation of cultures (30°C, 8~12 day except in the case of gelatin stab)	Czapek's agar	Faint growth with a slightly pinkish gray powdery aerial mycel. or only colorless thin growth. Reverse, light gray~white. Microscopical observation of aerial mycel. revealed a rather thick (about 1~2 μd.) long straight hyphae with close spirals on the tips.
	Starch-ammonium agar	Growth rather appearing restricted with thick grayish pink aerial mycel. Reverse, gray occasionally almost black. The morphology of aerial mycel. is the same as on Czapek's agar. Diastatic action is very weak.
	Glucose-asparagine agar	Growth covered with a thin gray powdery aerial mycel. Reverse, brown. Light brown soluble pigment is produced.
	Bouillon agar	Aerial mycel. never found on this medium. Wet grayish cream flat growth. Brown soluble pigment is produced.
	Glucose bouillon	Brown ring growth with white powdery aerial mycel. grows on the surface. Brown soluble pigment is produced. Reaction of broth slightly acidified.
	Czapek's solution (glucose is used as carbon sourse)	Little colonies with grayish aerial mycel. on the surface of broth, sometimes as pellicle. Brown soluble pigment is produced. Nitrite reaction is negative except in very few cases.
	Gelatin stab (10~20°C, 23 days)	Creamy growth with no aerial mycel. subsides into medium. No liquifaction. Brownish black soluble pigment is markedly produced around the growth.
	Potato plug	Dark creamy growth with brown soluble pigment.
	Carrot plug	No growth observed.
Utilization of carbon sources1)	Utilized	glucose, salicin
	Not utilized	xylose, arabinose, rhamnose, trehalose, lactose, raffinose, mannitol, inocitol

Note: 1) The agar removed from the basal medium reported by Pridham et al. described in *J. Bact.*, **56**, 107 (1948).

TABLE II
CULTURE CONDITIONS OF *STREPTOMYCES* sp. S-580

		Shaking culture	Submerged culture
Culture medium	Glucose	1.0%	1.0~2.0%
	Poly peptone	0.5 〃	0.5~1.0 〃
	Meat ext.	0.5 〃	0.5 〃
	NaCl	0.3 〃	0.3 〃
	pH	7.6 (NaOH)	7.6 (NaOH)
Inoculum		One loopful of spore from the slant cultured for seven days on Bennett's agar.	An amount of 200~400 ml of shaking broth cultured for 18~30 hrs. In the case of large scale, 40~70 l of submerged broth cultured for 24~48 hrs.
Culture temperature		27±1°C	28±1°C
Culture volume		One hundred ml in 500 ml of Sakaguchi's flask.	Seventy l in 100 l of fermentor. In the case of large scale 600 l in 1000 l of fermentor.
Mechanical conditions		Stroke: 15 cm Oscillation: 120 o.p.m.	Rotation: 200 r.p.m. Aeration: $0.5\ m^3/m^3 \cdot min$

TABLE III

EFFECT OF THE CULTURE FILTRATE OF *STREPTOMYCES* sp. S-580 ON THE GERMINATION OF RICE PLANT SEEDS

(I) In the case of the shaking culture.

	Culture temperature	Concentration of cult. filt. \ Culture age, days	2	3	4	5	6	7	8	9
Test - 1	27 ±1°C	1 ml × 10^{-2}	0	1	1	2	2	3	1	2
		2.5 ml × 10^{-3}	0	1	1	2	2	3	2	2
		1.25 ml × 10^{-3}	0	1	2	1	2	3	2	2
Test - 2	31 ±1°C	1 ml × 10^{-2}	0	0	1	0	1	0	1	0
		2.5 ml × 10^{-3}	1	0	0	0	0	0	0	0
		1.25 ml × 10^{-3}	1	0	0	0	0	0	0	0

Note: '0' shows no promoting effect against the control, '1' shows a slight promoting effect, '2' shows a strong promoting effect and '3' shows a very strong promoting effect. Thereafter, the same signs are used.

TABLE IV

EFFECT OF THE CULTURE FILTRATE OF *STREPTOMYCES* sp. S-580 ON THE GERMINATION OF RICE PLANT SEEDS

(II) In the case of submerged culture.

Test - 1	Cult. age, hrs.	14	20	26	32	38	44	50	56	62	68
	pH	7.0	7.0	7.0	6.3	6.5	6.9	6.8	7.1	6.9	7.6
	Glucose[1] %	—	0.8	—	0.5	—	0.2	—	0.1	—	0.1
	Activity, conc. of cult. filt. 1 ml × 10^{-2}	0	2	0	1	3	0	0	0	0	0
	5 ml × 10^{-3}	0	2	0	2	2	0	0	0	0	0
	2.5 ml × 10^{-3}	1	1	0	1	2	0	0	0	0	0
Test - 2	Cult. age, hrs.	56	62	68	74	80	86	92	98	104	110
	pH	6.9	6.8	7.1	6.7	6.8	7.0	7.5	7.5	7.8	7.8
	Glucose %	0.6	—	0.6	—	0.6	—	0.3	—	0.2	—
	Activity, conc. of cult. filt. 1 ml × 10^{-2}	0	0	0	1	2	0	1	1	1	0
	5 ml × 10^{-3}	0	0	1	1	1	1	1	1	1	1
	2.5 ml × 10^{-3}	0	0	1	0	2	1	1	1	1	1

Note: 1) Quantitative determination of glucose was carried out by the Shaffer-Harpman's method.

5) Microbiological characteristics of *Streptomyces* sp. S-580. *Streptomyces* sp. S-580 has been newly isolated from soil by the members of the antibiotic research group in this department. According to its microbiological characteristics which have been described by H. Sakai in this department as shown in Table I, this streptomyces is similar to *Streptomyces lavendulae*. However, there is obviously a difference between the two as follows; the former produced a brownish soluble pigment when it was cultured on the glucose-asparagine agar and in Czapek's solution whereas the latter did not produce such pigment under the same condition.

6) Culture conditions of *St.* sp. S-580. The culture conditions of *St.* sp. S-580 are described in Table II. In the case of shaking culture, activity of the culture filtrate in promoting the germination of rice plant seeds appeared in the middle or late-stage of the culture as shown in Table III—Test-1; however, when culture was carried out under rather high temperature (31±1°C) activity was not recognized as shown in Table 3—Test-2. In the case of submerged culture, as shown in Table IV—Test-1, activity appeared in a rather early-stage of the culture, thereafter disappeared rapidly. While, as shown in Table IV—Test-2, activity was only very weak in regard to the culture in which growth in the early-stage was so poor that abundant growth was attained at about fourty hours after inoculation whereas usually at about twenty hours.

7) Fxtraction procedure of the active crude syrup (A.C.S.) from the culture filtrate of *St.* sp. S-580. As a preliminary experiment, the extraction with ethyl ether and ethyl acetate and the adsorption with active carbon (Seisei-Shirasagi) were carried out on the culture filtrate of *St.* sp. S-580 harvested by the shaking culture. The extraction procedure was carried out as follows: (a) The culture filtrate, without pH adjustment, (usually, pH 8.6~8.8) was shaken with the same volume of solvent in the separating funnel for thirty minutes. (b) The solvent layer was separated and the residual water layer was adjusted to pH 3.0 with 1N HCl and the whole treatment was repeated. (c) Both solvent layers were combined and then evaporated *in vacuo*. The adsorption procedure was carried out as follows: (a) The culture filtrate, without pH adjustment, was shaken with 2% (W/V) active carbon in a shaking flask for thirty minutes. (b) The active carbon was separated by filtration and the filtrate was adjusted to pH 3.0 with 1N HCl and the whole treatment was repeated. (c) Both active carbons were combined and shaken with a twenty-fivefold (V/W) 75% acetone solution in a shaking flask for thirty minutes. (d) The active carbon was separated by filtration and shaken with a twenty-fivefold (V/W) 75% acetone solution 0.1N in respect of HCl under the same conditon. (e) The active carbon was separated by filtration and both filtrates were combined and neutralized with 1N NaOH and then evaporated *in vacuo*. As shown in Table V, the fraction of ethyl acetate-extraction showed high activity. (However, this activity was less than that of the culture filtrate and studies concerning this fact are now in progress.) While, sometimes, activity was only very weak in regard to the usual culture. Then, the culture filtrate having such low activity was shaken with 0.2% (W/V) active carbon for thirty minutes at pH 8.6 (non adjusted), 7.0, 5.0 and 3.0 (the latter three were adjusted with 1N HCl individually) and the active carbon was separated, and activity of the each filtrate was determined. As shown in Table VI, activity of the filtrate treated with active carbon at neutral and acidic side, could be increased. Therefore, it may be presumed that either some antogonists or inhibitors produced in such culture were adsorbed with active carbon at neutral and acidic side, especially at acidic side, consequently activity of the filtrate was recovered. Finally, the effect of pH adjustment of the culture filtrate in the case of extraction of the active fraction with ethyl acetate, was investigated. The culture filtrate was adjusted to pH 7.0 and 3.0 with 1N HCl, and shaken with the same volume of ethyl acetate in a separating funnel for thirty minutes and the solvent layer was separated. After that, the whole treatment was repeated twice, and all three solvent layers were combined and evaporated *in vacuo*.

TABLE V

PRELIMINARY EXPERIMENT ON THE EXTRACTION OF ACTIVE FRACTION FROM THE CULTURE FILTRATE OF *STREPTOMYCES* sp. S-580

Concentration of the test solution	Part	Fraction extracted with ethyl ether from the cult. filt.	Fraction extracted with ethyl acetate from the cult. filt.	Fraction eluted with acetone from the act. carbon adsorbed the cult. filt.
1 ml $\times 10^{-1}$	stem	0	0	0
	root	0	0	0
2.5 ml $\times 10^{-2}$	stem	0	1	2
	root	0	0	1
6.25 ml $\times 10^{-3}$	stem	0	2	1
	root	0	1	0
1.5625 ml $\times 10^{-3}$	stem	0	2	1
	root	0	2	1

TABLE VI

EFFECT OF ACTIVE CARBON-ADSORPTION ON THE ACTIVATION OF LESSER ACTIVE CULTURE FILTRATE OF *STREPTOMYCES* sp. S-580

Conc.	Part	Not treated	pH 3.0	pH 5.0	pH 7.0	pH 8.6
1 ml $\times 10^{-1}$	stem	1	0	1	2	0
	root	0	0	0	0	0
1 ml $\times 10^{-2}$	stem	1	0	2	2	0
	root	0	0	2	1	0
1 ml $\times 10^{-3}$	stem	0	1	0	1	0
	root	1	1	1	1	0
1 ml $\times 10^{-4}$	stem	1	2	1	1	0
	root	1	2	1	1	0

TABLE VII

EFFECT OF pH ADJUSTMENT OF THE CULTURE FILTRATE OF *STREPTOMYCES* sp. S-580 IN THE CASE OF THE EXTRACTION OF ACTIVE FRACTION WITH ETHYL ACETATE

Conc.	Part	pH 3.0	pH 7.0	pH 8.6
1 ml $\times10^{-1}$	stem	1	1	2
	root	2	1	2
1 ml $\times10^{-2}$	stem	1	1	2
	root	1	1	2
1 ml $\times10^{-3}$	stem	1	1	0
	root	2	2	1
1 ml $\times10^{-4}$	stem	1	2	0
	root	1	2	1
1 ml $\times10^{-5}$	stem	2	1	0
	root	2	2	1

As shown in Table VII, all fractions extracted at varied pH used in this experiment, showed activity in promoting the germination of rice plant seeds, especially at acidic side, the active fraction might be extrated more successfully. Consequently, the most successful procedure for extraction of the active crude syrup (A.C.S.), the deep reddish-orange colored heavy syrup, from the culture filtrate of *St.* sp. S-580 is outlined in Fig. 2. Furthermore, active factors have been isolated from A.C.S. and their properties, structures etc. will soon be reported.

8) **Effect of A.C.S. on the germination of rice plant, barley, wheat and oats seeds.** As indicated in Table VIII, A.C.S. strongly promoted the germination of rice plant seeds in the concentration range of $1\,\mathrm{ml}\times10^{-6}\sim10^{-8}$, although in the concentration of $1\,\mathrm{ml}\times10^{-7}$ was most effective. However, activity of A.C.S. is less than that of the culture filtrate in the concentration of $1.25\,\mathrm{ml}\times10^{-3}$ as shown in Table IX.

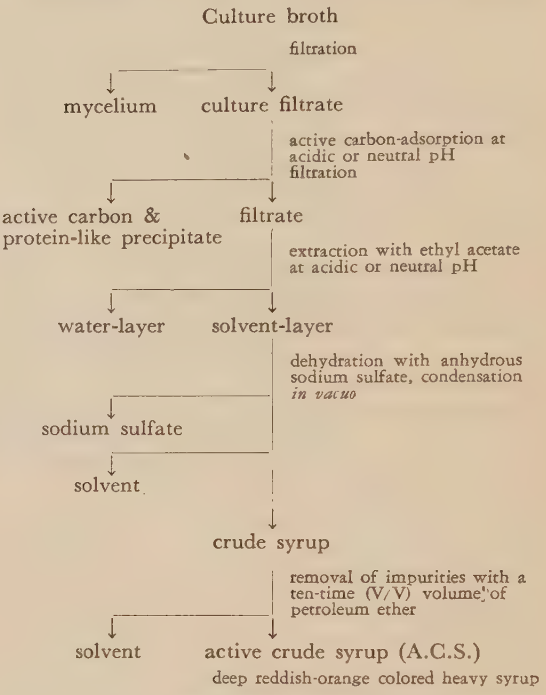
Culture broth
filtration
mycelium
culture filtrate
active carbon-adsorption at acidic or neutral pH filtration
active carbon & protein-like precipitate
filtrate
extraction with ethyl acetate at acidic or neutral pH
water-layer
solvent-layer
dehydration with anhydrous sodium sulfate, condensation *in vacuo*
sodium sulfate
solvent
crude syrup
removal of impurities with a ten-time (V/V) volume of petroleum ether
solvent
active crude syrup (A.C.S.)
deep reddish-orange colored heavy syrup

FIG. 2. Extraction procedure of A.C.S. from the Culture Broth of *STREPTOMYCES* sp. S-580.

TABLE VIII

EFFECT OF A.C.S. ON THE GERMINATION OF RICE PLANT SEEDS

Ten days after start of experiment under the dark condition, incubated at 18°C, diluted with distilled water.

	Control[1]	1 ml $\times10^{-3}$	〃 $\times10^{-4}$	〃 $\times10^{-5}$	〃 $\times10^{-6}$	〃 $\times10^{-7}$	〃 $\times10^{-8}$	〃 $\times10^{-9}$	〃 $\times10^{-10}$
Stem cm (%)	1.7	2.1 (24)[2]	2.1 (24)	2.0 (18)	2.1 (24)	2.2 (29)	2.0 (18)	1.9 (12)	1.9 (12)
Root cm (%)	3.8	2.4 (−37)	5.1 (34)	5.0 (32)	5.3 (39)	5.6 (48)	5.2 (37)	5.0 (32)	4.8 (26)
Prophyll formation	—	2	2	1	2	2	0	0	0
Crown root formation	—	1	2	1	1	2	1	1	1
Root hair formation	—	0	1	1	1	2	1	0	0
Interim germination state	—	0	1	0	1	2	1	0	0

Note: 1) Treated with distilled water.
2) Figures in the brackets indicate the promoting ratio againt control; minus numbers represent the inhibiting ratio.

TABLE IX

COMPARISON BETWEEN THE ACTIVITY OF A.C.S. AND THE CULTURE FILTRATE OF *STREPTOMYCES* sp. S-580 FOR THE PROMOTING EFFECT ON THE GERMINATION OF RICE PLANT SEEDS

	A.C.S.	Culture filt. of *St.* sp. S-580
Concentration	$1\ \text{ml}\times10^{-7}$	$1.25\ \text{ml}\times10^{-3}$
Promoting ratio of stem elongation %	29	67
Promoting ratio of root elongation %	48	112
Promoting degree of prophyll formation	2	3
Promoting degree of crown root formation	2	3
Promoting degree of root hair formation	2	3
Promoting degree of interim germination state	2	3

Studies concerning this fact are now in progress. Effect of A.C.S. on the germination of barley (Sekitori-Saitama-No. 1), wheat (Norin-No. 61) and oats (Victory-No. 1) seeds, which were all harvested in 1956, are shown in Table X. In the concentration range of $1\ \text{ml}\times10^{-5}\sim10^{-7}$, A.C.S. strongly promoted the germination of barley and oats seeds. However, A.C.S. did not promoted the germination of wheat, which was probably slightly inhibited in rather high concentration range.

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TABLE X

EFFECT OF A.C.S. ON THE GERMINATION OF BARLEY, WHEAT AND OATS SEED

Five days after start of the experiment under the dark condition, incubated at 18°C, diluting with distilled water.

	Barley		Wheat		Oats	
	stem cm (%)	root cm (%)	stem cm (%)	root cm (%)	stem cm (%)	root cm (%)
Control[1)]	1.05 (0)	2.35 (0)	2.55 (0)	4.85 (0)	2.50 (0)	2.70 (0)
$1\ \text{ml}\times10^{-4}$	1.35 (29)	2.70 (15)	2.55 (0)	4.25 (−12)	2.65 (6)	2.55 (−6)
〃 $\times10^{-5}$	1.65 (57)	3.50 (49)	2.75 (8)	4.75 (−2)	3.65 (46)	3.70 (37)
〃 $\times10^{-6}$	1.55 (48)	2.90 (23)	2.60 (2)	4.50 (−7)	3.70 (48)	3.85 (43)
〃 $\times10^{-7}$	1.50 (43)	2.85 (21)	2.40 (−6)	4.80 (−1)	3.50 (40)	3.45 (31)

Note: 1) Treated with distilled water.
2) Figures in the brackets indicate the promoting (minus figures represent the inhibiting) ratio against that of the control.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 2, p. 98~103, 1958]

Germination Promotant for Plants Seed, produced by Microorganisms

Part III. Germination Promotant for Rice Plant Seeds, produced by *Streptomyces* sp. S-580. Isolation and Structure of an Active Neutral Crystal (Factor-E)*

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Received November 5, 1957

A neutral crystalline substance (Factor-E), which is effective for the germination of rice plant seeds at the dilution of $10^{-5} \sim 10^{-7}$, was isolated from the active crude syrup extracted from the culture filtrate of *Streptomyces* sp. S-580. And Factor-E has been proved by hydrolysis and synthesis to be L-prolyl-L-valine anhydride (diketopiperazine), which has been proved to be the new diketopiperazine.

INTRODUCTION

In the previous paper[1], it has been reported that the active crude syrup (A.C.S.) showing strong activity in promoting the germination of rice plant seeds, was extracted with ethyl acetate from the culture filtrate of *Streptomyces* sp. S-580. Furthermore, in the short communication[2], it has also been reported that an active neutral crystal (Factor-E), which is effective at the dilution of $10^{-5} \sim 10^{-7}$ for the germination of rice plant seeds, was isolated from the active crude syrup (A.C.S.) by a chromatographic technique using aluminium oxide, and as the result of investigations of its properties, it was pressumed to be prolylvaline anhydride (diketopiperazine). Furthermore, its properties were investigated in detail and synthesis of L-prolyl-L-valine anhydride (diketopiperazine) from L-proline and L-valine by the N-carbobenzoxy method, was carried out. As compared with the synthesized sample, Factor-E is shown to be L-prolyl-L-valine anhydride (diketopiperazine). Formerly, it had been reported by Abderhalden et al.[3] that *l*-prolyl-*d*-valine anhydride was isolated from the hydrolysate of pig bristle keratin. However, the descriptions concerning properties of this substance were insufficient and the melting point of this substance was significantly higher than that of Factor-E. So, it appears most probable that the former was either a diasteroisomer or a polymer of the latter. Consequently, Factor-E has been proved to be the new diketopiperazine having the above mentioned structure. Furthermore, anther crystalline substance, by which the activity of Factor-E could be increased synergistically, was isolated from the same active crude syrup, and its properties, structure etc. will soon be reported.

EXPERIMENTAL AND RESULTS

1) Isolation of Factor-E from the active crude syrup (A.C.S.). Isolation of Factor-E from the active crude syrup (A.C.S.) was carried out by a chromatographic technique using aluminium oxide (standardized for chromatographic adsorption analysis acc. to Brockmann) as follows: (a) aluminium oxide was introduced into the glass tube with diameter 2.2 cm to make the column with high 24 cm, (b) then 25 ml of

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* Presented at the Meeting of the Agricultural Chemical Society of Japan held in Tokyo, October 26, 1957.

1) Y. Koaze, this Bulletin, **22**, 91 (1958).

2) Y. Koaze, *ibid.*, **21**, 197 (1957).

3) E. Abderhalden and E. Komm, *Z. physiol. Chem.*, **132**, 4 (1924).

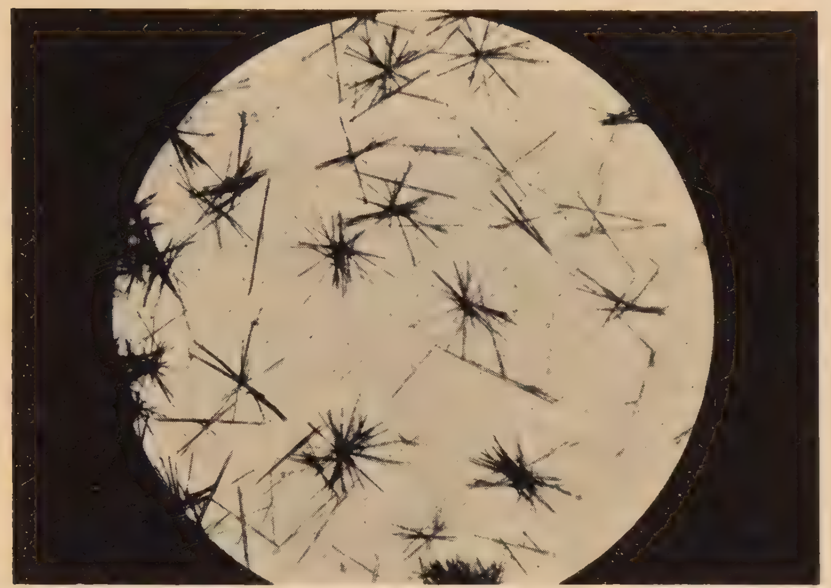

FIG. 1. The Crystalline Form of Factor-E. (magnification ×150)

TABLE I

EFFECT OF FACTOR-E ON THE GERMINATION OF RICE PLANT SEEDS

Ten days after start of experiment under the dark condition, incubated at 18°C, diluted with distilled water containing 50 mcg/ml of ammonium sulfate.

	Control[1]	1 gr × 10^{-4}	1 gr × 10^{-5}	1 gr × 10^{-6}	1 gr × 10^{-7}	1 gr × 10^{-8}
Stem cm (%)	1.52	1.71 (13)[2]	1.78 (17)	1.73 (14)	1.77 (16)	1.61 (6)
Root cm (%)	2.98	3.41 (14)	3.86 (30)	4.05 (36)	4.32 (45)	3.77 (27)
Prophyll formation ratio %	20	46	33	41	54	42
Crown root formation ratio %	0	18	22	37	38	10
Root hair formation ratio %	0	0	15	4	8	0

Note: 1) Treated with distilled water containing 50 mcg/ml of ammonium sulfate.
2) Figures in the brackets show the promoting ratio against the control.

A.C.S. dissolved in 25 ml of ethanol was charged into this column and flowed out at the flow rate of 1.5~2.0 ml/min. and eluted with a sufficient volume of ethanol at the same flow rate. (c) The colorless and pale yellow fractions, flowed out before the deep brown fraction, were combined and evaporated *in vacuo*. (d) Presently a large amount of needles appeared in the brown syrup and they were then dissolved in ethanol and decolored with a small amount of active carbon. (e) Repeated crystallizations from ethanol gave colorless needles (Factor-E) as shown in Fig. 1.

2) Effect of Factor-E on the germination of rice plant seeds. Fujisaka-No. 5 harvested in 1956 was used in this experiment. Condition on the germination of rice plant seeds, determination of the activity in promoting the germination of rice plant seeds and preparation of the test solution were the same as described in the previous papers[1,4]. However, the test solutions of Factor-E were diluted with sterilized distilled water containing 50 mcg/ml of ammonium sulfate. As indicated in Table I, Factor-E

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[4] Y. Koaze et al., *J. Agr. Chem. Soc. Japan*, **31**, 338 (1957).

showed the promoting effect on the germination of rice plant seeds in the concentration range of $1\ \mathrm{gr} \times 10^{-5} \sim 10^{-7}$, although in the concentration of $1\ \mathrm{gr} \times 10^{-7}$ was most effective. However, activity of Factor-E is not only less than that of the culture filtrate of *Streptomyces* sp. S-580, but also less than that of A.C.S.. Recently, another active crystalline substance, by which activity of Factor-E could be increased synergistically, was isolated from the same active crude syrup, and its properties, structure etc. will soon be reported.

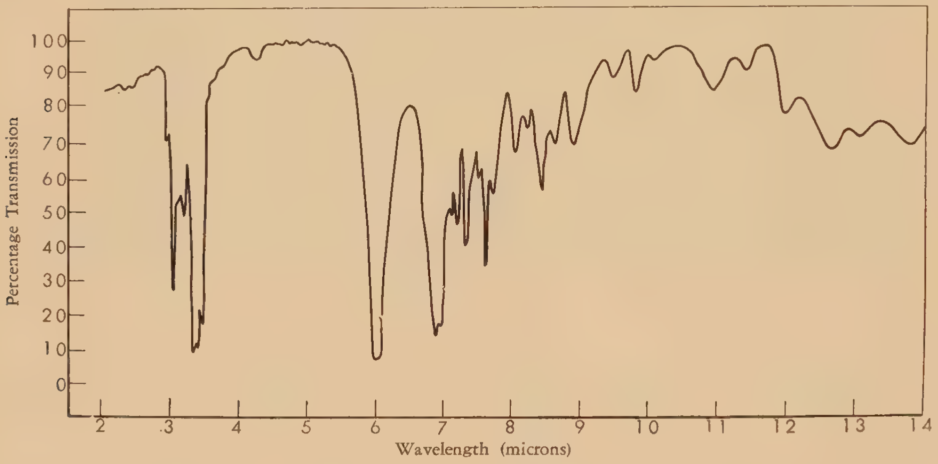

FIG. 2. Infrad-red Spectrum of Factor-E in Nujol Mull.

3) Properties of Factor-E. Factor-E is a neutral colorless needle (from ethanol), m.p. 191~193°C (the m.p. described in the short communication[2] is corrected as mentioned above); $[\alpha]_D^{20} = -160.4°$ (in ethanol); soluble in acetone, alcohols, benzene, chloroform, ethyl acetate and water, very slightly soluble in ethyl ether but insoluble in *n*-hexane and petroleum ether. Analytical data of this substance was as follows: *Anal.* Calcd. for $C_{10}H_{16}O_2N_2$: C, 61.20: H, 8.22: N, 14.28: mol wt, 196.24, Found: C, 61.06, 61.18: H, 8.20, 8.22: N, 14.05: mol wt, (by the micro-Rast method dissolving in camphor), 207.19. Therefore, this substance was proved to have the empirical formula: $C_{10}H_{16}O_2N_2$. The UV-spectrum of this substance in methyl alcohol showed no significant absorption in the wavelength range of 221~400 mμ, but showed a strong end-absorption. The infra-red spectrum of this substance in Nujol mull is shown in Fig. 2. Both the ninhydrin reaction and the biuret reaction on this substance were negative.

4) Hydrolysis of Factor-E. Factor-E was hydrolyzed with fourtyfold (V/W) 6N HCl at 120±2°C for eighteen hours in the sealed glass tube. In the following experiments, the hydrolysate was examined after removal of most of the acid by repeated evaporation with water, over solid NaOH *in vacuo*. (a) Detection of amino acids: The paper chromatographic method (filter paper, Toyoroshi-No. 5; solvent, *n*-butanol: acetic acid: water=4:1:5 or phenol: water=4:1; ascending for twenty to twenty-four hours at room temperature; development of spot, 0.1% ninhydrin in *n*-butanol saturated with water) was used. As the result, the hydrolysate gave only two known amino acids, proline and valine. (b) Determination of the configuration of these two amino acids: Activity of D-Amino acid oxidase (prepared from pig kidney in our laboratory) towards the hydrolysate, determined by the manometric method, was negative. Therefore, these two amino acids have the L-configuration. (c) Quantitative determination of these two amino acids: The microbioassay (microorganisms used for assay, *Leuc. mesenteroides* for L-valine and *Leuc. citrovorum* for L-proline; culture, 36 C/72 hours) and the photometric method[5] (wavelength used for determination, 570 mμ for L-valine and 430 mμ for L-proline; determination was carried out in a mixture of these two amino acids) were applied. As the result, the hydrolysate of 1 mol of Factor-E consists of 1 ml of L-proline and 1 mol of L-valine.

5) Synthesis of L-prolyl-L-valine anhydride (diketopiperazine). At first, L-prolyl-L-valine (or its methyl ester) was synthesized from L-proline (Wako-junyaku-Kogyo Co. Ltd.) and L-valine (Takara Pharma-

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5) S. Moore and W. H. Stein, *J.B.C.*, **211**, 907 (1954).

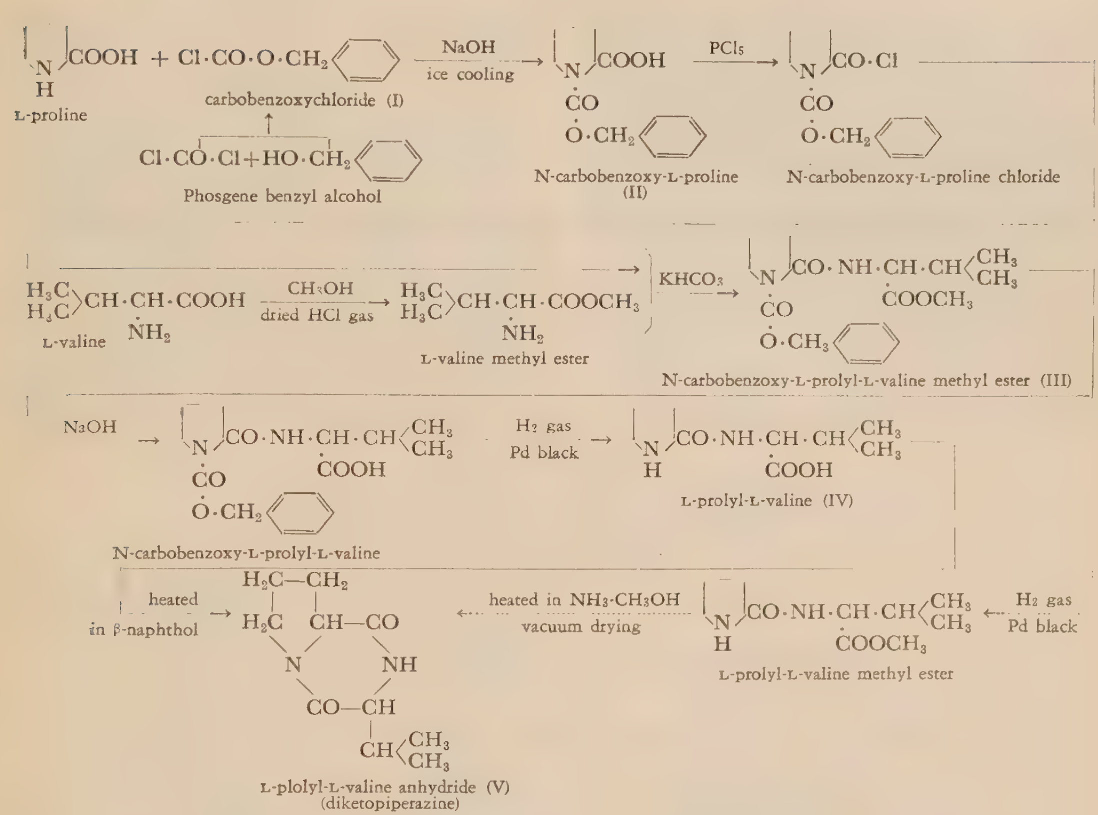

FIG. 3. Outlines of the Synthesis of L-Prolyl-L-Valine Anhydride (Diketopiperazine)

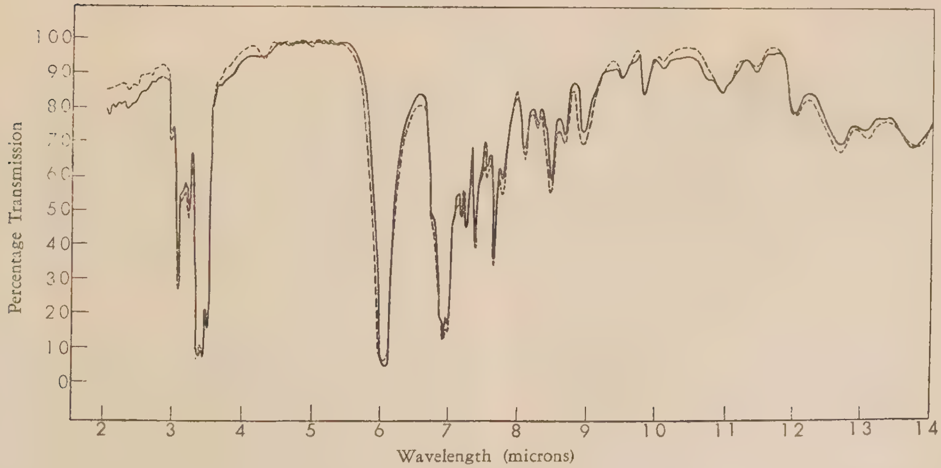

FIG. 4. Comparison between the Infra-red Spectrum of Factor-E and Synthesized L-Prolyl-L-valine Anhydride (diketopiperazine) in Nujol Mull.

—— Synthesized L-prolyl-L-valine anhydride (diketopiperazine)
-·--- Factor-E

ceutical Co. Ltd.) by the N-carbobenzoxy, method[6], then, ringed through dehydration to L-prolyl-L-valine anhydride (diketopiperazine). The outlines of synthesis are shown in Fig. 3, and mentioned as follows: (a) carbobenzoxychloride (I) was formed from phosgene and benzyl alcohol by the usual way[7]. (b) N-carbobenzoxy-L-proline (II) was formed from (I) and L-proline by the method described by Abderhalden et al.[8]. (c) N-carbobenzoxy-L-prolyl-L-valine methyl ester (III) was produced from acid chloride of (II) and L-valine methyl ester formed by the usual way[9] from L-valine. (d) (III) was saponified and then hydrogenated with gaseour hydrogen at atmospheric pressure and room temperature in the presence of active Pd. As the result, L-prolyl-L-valine (hydrate) (IV) was formed. (e) At last, L-proly-L-valine anhydride (diketopierzaine (V) was formed through dehydration of (IV) by the method described by Lichenstein[10]. (f) On the other hand (III) was hydrogenated and then ringed by the usual way[11] to (V). Repeated crystallizations from ethanol gave colorless needles, m.p. 192～194°C and $[\alpha]_D^{24}=-161.9°$ (in ethanol). Analytical data of this substance was as follows: Found: C, 60.95: H, 8.00: N, 14.28: mol wt (by the micro-Rast method dissolving in camphor), 208.5. The ninhydrin reaction on this substance was negative and the infra-red spectrum of this substance in Nujol mull is shown in Fig. 4.

Table II

Comparison between the Properties of Factor-E, Synthesized L-Prolyl-L-Valine Anhydride (diketopiperazine) and *l*-Prolyl-*d*-Valine Anhydride reported by Abderhalden et al.[3]

		Factor-E	Synthesized L-prolyl-L-valine anhydride (diketopiperazine)	*l*-prolyl-*d*-valine anhydride reported by Abderhalden et al.[3]
Anal.	C	61.06, 61.18	60.95	61.03
	H	8.20, 8.22	8.00	8.17
	N	14.05	14.28	14.75
mol wt.		207.19	208.5	no description
m.p.		191～193°C	192～194°C	250～252°C
$[\alpha]_D$		−160.4° (20°C, ethanol)	−161.9° (24°C, ethanol)	drehte stark nach links (Alkohol)
Ninhydrin reaction		negative	negative	negative
Mixed m.p.		gave no depression		

DISCUSSION

From evidences mentioned as follows, it seemed most probable that Factor-E possessed the structure as L-prolyl-L-valine anhydride (diketopiperazine): (a) Factor-E proved to have the empirical formula $C_{10}H_{16}O_2N_2$, (b) and hydrolysate of 1 mol of this substance consists of 1 mol of L-proline and 1 mol of L-valine, (c) while, both ninhydrin and biuret reactions on this substance were negative. (d) Infra-red spectrum of this substance showed characteristic absorption[12,13] as diketopiperazine. Further more, from evidences described as follows, the above mentioned presumption was authenticated: (a) properties and infrared spectrum of Factor-E were clearly identical with that of the synthesized sample, (b) and a mixed melting point with synthesized sample gave no depression.

On the other hand, it had formerly been reported by Abderhalden et al.[3] that *l*-prolyl-*d*-valine anhydride was isolated from the hydrolysate of pig bristle keratin. Properties of this substance reported by Abderhalden et al.[3] are described in Table II. From description of this Table, it seems most probable that this substance is distinct from Factor-E. The former may be either a diasteroisomer or a polymer of the latter. Consequently, Factor-E has been proved to be the new diketopiperazine having the above mentioned structure.

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[6] R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948).

[7] S. Akabori and S. Mizushima, Chemistry of Protein (Kyoritsu Publishing Co. Ltd.), (1) p. 657 (1954).

[8] E. Abderhalden et al., *Fermentforschung*, **13**, 591 (1933).

[9] S. Akabori and S. Mizushima, *ibid*, p. 459.

[10] N. Lichenstein, *J.A.C.S.*, **60**, 560 (1938).

[11] S. Akabori and S. Mizushima, *ibid*, p. 460.

[12] S. Mizushima et al., *J.A.C.S.*, **72**, 3490 (1950).

[13] M. Tsuboi et al., *Bull. Chem. Soc. Japan*, **22**, 255 (1949).

Acknowledgements. The author wishes to express his sincere thanks to Prof. K. Sakaguchi, Univ. of Tokyo for his constant guidance throughout this work, and also to Prof. Y. Sumiki, Prof. T. Asai and Prof. M. Matsui, Univ. of Tokyo for their kind advice and suggestions extended throughout this work. Thanks are also due to Prof. S. Akabori, Univ. of Osaka for his generous supply of DL-proline, and to Mr. I. Matsumoto, Banyu Pharmaceutical Co. Ltd. for his kind supply of phosgene, further to Mr. K, Sugimura, Food Research Institute and Mr. J. Kirimura, Sericultural Experiment Station, Ministry of Agriculture and Forestry, for their help in the microbioassay. The micro analysis and infrared spectrum were carried out at this department.

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Biochemical Studies of *Piricularia oryzae*

Part V. Behavior towards DL-Norleucine and DL-Alanine.*

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Received November 13, 1957

In the current experiment, the conversion of DL-norleucine and DL-alanine to their respective α-keto analogue by *Piricularia oryzae* was confirmed by the separation of 2,4-dinitrophenylhydrazone of either keto acid. Although the formation of keto acid from norleucine was distinctly recognized both in the presence and the absence of arsenite, the accumulation of pyruvic acid from alanine was only found in the presence of arsenite, as it has been observed with some microorganisms. In addition, the preferential utilization of alanine to a proper carbon source was identified, which may provide a step towards the investigation of the nutritional relationship between the blast fungus and the host plant.

It is apparent that the study of the biochemical behavior of *Piricularia oryzae* towards nitrogenous compounds, especially amino acids, is of fundamental importance in order to prevent the host plant from the blast disease. From such a point of view, the utilization process of several amino acids by this fungus has been investigated by the authors[1,2,3,4,5] and the conversion of the amino acid to respective α-keto analogue was recognized. In current experiments, DL-norleucihe and DL-alanine were taken as the amino acid to be tested. As already reported, the six carbon amino acids such as leucine[1] and isoleucine[5] have been examined in regard of their conversion to respective α-keto acid and thus the remaining six carbon amino acid, that is, norleucine was naturally taken into question as the supplement, although its natural occurrence is still questionable at present[6], in spite of the earlier works of Abderhalden et al.. On the other hand, the general metabolic significance of alanine has been well known and it was reported by Tomizawa[7] and Otani[8] that this amino acid is effective as a nitrogen source in the same extent as asparagine, aspartic acid, glutamic acid, and glycine, but metabolic conversion of these amino acids was unstated. Tanaka[9], reported that glutamic acid could be utilized preferentially to a proper carbon source by this fungus and proceeded that this fact might be interpreted as one of the biochemical mechanisms involved in the invasion of blast fungus in the host plant and its further growth. Similar results have been obtained with alanine by the authors.

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* Presented in part at the Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, April 1, 1956.

[1] Y. Nakamura and T. Shimomura, *J. Agr. Chem. Soc.* Japan, **27**, 694, (1953).

[2] Y. Nakamura, T. Shimomura and T. Sugimoto, *Memoirs, Fac. Agr., Hokkaido Univ.*, **2**, 30, (1955).

[3] T. Shimomura and Y. Nakamura, This Bulletin, **19**, 178, (1955).

[4] Y. Nakamura and T. Shimomura, the Annual Meeting of Agr. Chem. Soc., Tokyo, April 1, (1955).

[5] T. Shimomura and Y. Nakamura, part VI of this series.

The present communication records the utilization process of both amino acids just mentioned, the separation and identification of either keto acid and the preferential utilization of alanine to a proper carbon source, D-glucose.

EXPERIMENTAL AND RESULTS

Incubation—Two mycelial felts were incubated at 27–8° in 60 ml of complete medium[3] containing 100 mg of DL-norleucine or DL-alanine in place of sucrose. In the inhibition experiment, 5 ml of M/20 potassium arsenite was substituted for 5 ml of water. Ammonia- and amino-nitrogen in the culture filtrate during the incubation were determined as previously reported (Fig. 1 and Fig. 2). The same determinations were made with the culture medium consisting of 30 ml of M/15 phosphate buffer (pH 6.9), 100 mg of DL-alanine and 30 ml of water and similar results as in the complete medium just mentioned were obtained (Fig. 3). This fact indicates that it is possible to carry out such experiment in a phosphate buffer medium without the addition of other inorganic salt. The determination of pyruvic acid accumulated in the phosphate buffer medium was made by a modification of the Friedeman-Haugen method by Shimizu[10] (Fig. 4).

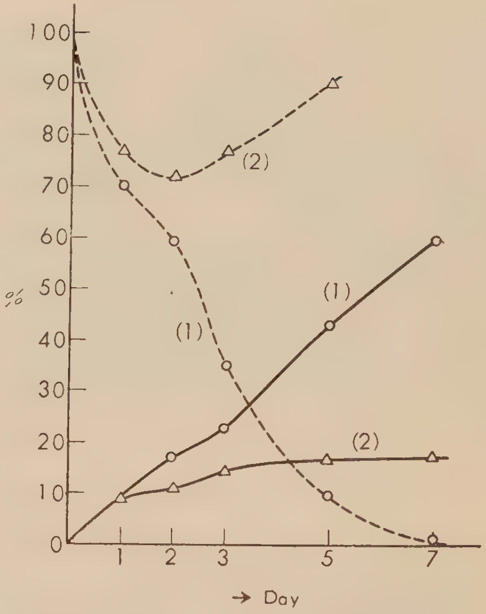

FIG. 1. Ammonia- and Amino-Nitrogen from DL-Norleucine by *P. oryzae*.

(1), DL-norleucine only; (2), DL-norleucine+arsenite. Full line expresses ammonia-nitrogen and dotted, amino-nitrogen. Values are indicated as percentage to theoretical amount of nitrogen of norleucine added as supplement. Incubation condition, see text.

In order to ascertain whether *P. oryzae* is able to utilize preferentially alanine to normal carbon source, this fungus was cultured at 27–8° in 30 ml of the medium containing 150 ml of DL-alanine, 792.5 mg of D-glucose and the same amount of inorganic salts and growth-factors as in the complete medium. The amount of carbon involved in present medium corresponded to that of 900 mg of sucrose which had been used as carbon source in normal cultivation. It was

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6) R. Consden, A. H. Gordon, A. J. P. Martin, O. Rosenheim and R. L. M. Synge, *Biochem. J.*, **39**, 251, (1945).

7) T. Tomizawa, *Ann. Phytopatholog. Soc. Japan*, **17**, 113, (1953).

8) Y. Otani, *Ann. Phytopatholog. Soc. Japan*, **17**, 9, (1952).

9) S. Tanaka, *Ann. Rep. Co-operat. Research (Agr.)*, Minist. Educat., **1956**, 92.

10) F. Egami et al., Standard Methods of Biochem. Experiments, Bunkodo, Tokyo, (1953), P. 36.

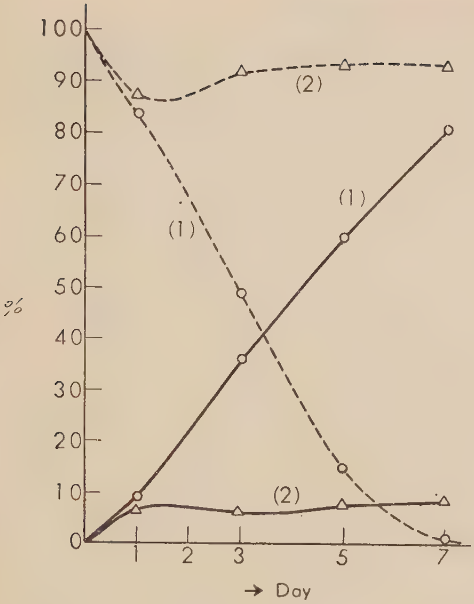

FIG. 2. Ammonia- and Amino-Nitrogen from DL-Alanine (in complete medium).

(1), DL-alanine only; (2), DL-alanine+arsenite. Expressions are the same as in Fig. 1.

already observed by Otani[11] that glucose was equally available for this fungus as sucrose. At appropriate intervals, ammonia- and amino-nitrogen, remaining glucose and the dry weight of mycelium were estimated. The decrease of the amount of detectable amino-nitrogen was expressed in per cent as alanine consumed. The decrease of the amount of alanine due to deamination was also involved in the data, but the formation of ammonia was far smaller throughout the present iucubation, that is, within the range of 0.69% to 2.52%. The results are presented in Table I.

Separation and Identification of Keto Acids—Two mycelial felts were incubated in 40 ml of the medium which consisted of 50 mg of DL-norleucine, 20 ml of M/15 phosphate buffer (pH 6.9), 5 ml of M/20 potassium arsenite and 15 ml of distilled water. Two hundred and fourty-five millilitre of a four-day old culture filtrate was concentrated under diminished pressure at pH 8.0~8.2 and extracted with ether for three hours, followed by acidic ether extraction for five hours. The aqueous solution of the residual oily substance from the latter ether-extract was added with a small amount of 2 N hydrochloric acid and reconcentrated under 14 mm Hg at about 45°. The treatment of the distillate with 2,4-dinitrophenylhydrazine gave a yellow crystal mass which melted at about 130°. Recrystallization with acidic dilute ethanol resulted in a long yellow needle of m.p. 148~9° (Found: C, 46.31; H, 4.38. Calcd. for $C_{12}H_{14}O_6N_4$: C, 46.45; H, 4.51.). In paper chromatography using butanol- 3% ammonia solvent, only one spot of $R_F=0.81\sim0.83$ was observed. This melting point is in good accordance with that of 2,4-dinitrophenylhydrazone of α-keto-caproic acid as

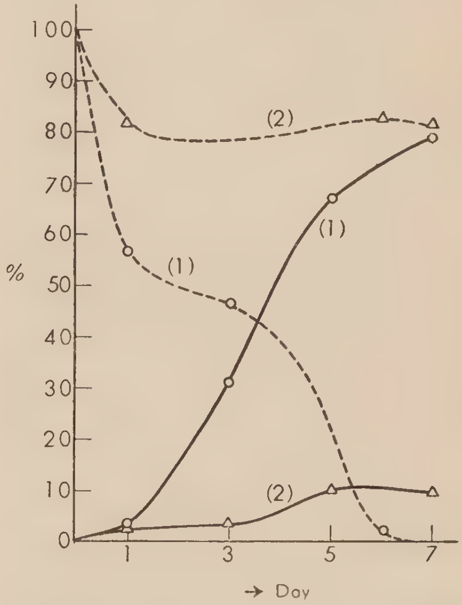

FIG. 3. Ammonia- and Amino-Nitrogen from DL-Alanine (in phosphate buffer medium, pH 6.9).

Expressions are the same as in Fig. 2.

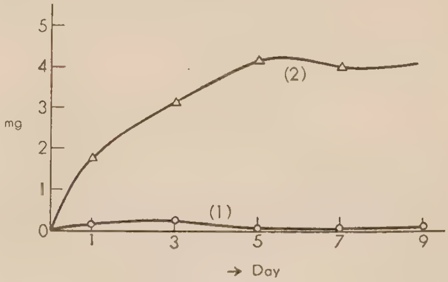

FIG. 4. Accumulation of Pyruvic Acid from DL-Alanine.

Values express the amount of pyruvic acid in 60 ml of phosphate buffer medium. (1), indicates the incubation added with no arsenite, and (2), added with arsenite.

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[11] Y. Otani, *Ann. Phytopatholog. Soc. Japan*, **17**, 119, (1953).

TABLE I. UTILIZATION OF ALANINE AND GLUCOSE

Day	1	3	5	7	10	12	14	17	20	22
Alanine consumed (%)	3.69	13.46	18.77	27.09	24.53	26.15	28.57	34.52	33.06	45.74
Glucose consumed (%)	3.02	5.01	5.01	8.61	7.37	17.46	21.87	26.50	44.09	82.36
Dry weight of mycelium (mg)	—	5.0	11.9	32.1	48.6	84.0	83.6	98.0	137.4	174.6

Present medium contained 150 mg of DL-alanine and 792.5 mg of D-glucose per flask. Figures express the average value of two flasks.

described by Krebs[12]. Yield, Ca. 2.5 mg. From the residual solution remaining in the distillation flask a small amount of the same hydrazone was recovered. The same 2,4-dinitrophenylhydrazone was also obtained from the medium without arsenite, although the separation procedure was a little more difficult and its yield was unsatisfactory, compared to the case in which arsenite was added as just mentioned.

The separation and identification of pyruvic acid derivable from alanine was far easier, compared to the separation experiments conducted so far with other several amino acids. The condition of incubation was the same as in the case of norleucine. An amount of 110 ml of the filtrate on the fifth day of incubation, which was strongly positive for Rothera's test, was treated in the same way as just described above. The 2,4-dinitrophenylhydrazone prepared from the acidic ether extract melted at 216~7°, on being recrystallized from ethanol solution and gave no depression in mixed melting point determination with an authentic sample. Yield, Ca. 7 mg. In paper chromatography (butanol-3% ammonia solvent; room temperature; ascending method) only one spot of $R_F=0.28\sim0.30$, i.e., anti-type of this hydrazone[13] was observed. The separation of this keto acid from the medium without arsenite resulted in fail, as it was expected from the negligible Rothera's reaction of the filtrate. This fact is only characteristic of alanine among the ten amino acids used, that is, L-leucine, DL-valine, L-aspartic acid, L-phenylalanine, DL-methionine, glycine, DL-norleucine, DL-isoleucine, DL-alanine and DL-α-aminobutyric acid, as a supplement in this series.

DISCUSSION

The formation of either α-keto acid from leucine[1] and isoleucine[5] by *Piricularia oryzae* has already been established by the authors. This led us to attempt to confirm such metabolic conversion with the remaining six carbon amino acid, that is, norleucine and thus, the formation of α-ketocaproic acid from this amino acid was successfully recognized both in the presence and absence of arsenite. In the absence of arsenite this amino acid was easily deaminated (Fig. 1) as was observed with other natural amino acids and, in addition, norleucine was found to be utilized in the form of free amino acid itself, that is, 20~23 per cent of the amino acid added up to the second day of incubation and 40~47 per cent up to the seventh day. This experimental result is somewhat different from the cases conducted with leucine[1] and valine[3], in which only a slight amount of each amino acid was utilized, not via its conversion to corresponding keto acid. In spite of uncertain natural occurrence of norleucine, the nutritional significance for some microorganisms has been examined by several investigators: growth-promoting effect for hiochi-bacteria[14], brewer's yeast[15], and *Neurospora* mutant[16] and, to the contrary, inhibitory effect for *E. coli*[17,18]. In addition, its role in transamination[19,20,21] and in oxidation by amino acid oxidase[22] was also observed. The present work seems to support further evidence that this amino acid is as much available as other natural amino acids, at least for some microorganisms. The amino nitrogen in the culture filtrate added with arsenite, however, was observed to increase again after the third day, in spite of the almost constant amount of ammonia produced. A similar tendency was observed in the case of α-aminobutyric acid[5] and rarely in the case of alanine as a supplement. This may be due to the production of endogenous amino acids, but at present no any data explaining thoroughly such observation are available.

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[13] T. Moriwaki, M. Katsuki and S. Tanaka, *J. Chem. Soc. Japan*, **76**, 1367, (1955).

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[15] C. Rainbow, *Chem. Abstr.*, **47**, 2261, (1953).

[16] R. W. Barratt and Wm. Ogata, *Am. J. Botany*, **41**, 763, (1954).

[17] D. Rowley, *Nature*, **171**, 80, (1953).

[18] W. Kellner and Hanna Hellmuth, *Chem. Abstr.*, **49**, 13355 (1955).

[19] J. H. Wiame and R. Storck, *Chem. Abstr.*, **47**, 5487, (1953).

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[22] Y. Otani, *Ann. Phytopatholog. Soc. Japan*, **18**, 159 (1954).

The nutritional availability of alanine as a nitrogen source for blast fungus was shown by Tomizawa[7], and Otani[22] has reported that amino acid oxidase secreted by present fungus is able to attack alanine. The present investigation was undertaken to examine the metabolic significance as a carbon source and thus the conversion of this amino acid to its α-keto analogue. Sufficient utilization of alanine is proved from Fig. 2, and the same utilization process was also found in the phosphate buffer medium (Fig. 3). From this result it is established that it is possible to carry out such an experiment just mentioned, in the phosphate buffer, if necessary. The absorption and utilization in the form of alanine itself was 14.8 per cent and 25.4 per cent on the third and the fifth day, respectively, but in the presence of arsenite such utilization process was almost negligible, although seven to eight per cent ammonia was produced up to the third day. It is noteworthy that the accumulation of pyruvic acid was detected only in the presence of arsenite and that in the absence of this inhibitor approximate one-fortieth of the amount in the former case was estimated (Fig. 4). Similar results were obtained in the investigation of the aerobic degradation of alanine and glucose by *E. coli* and *Clost. Perfringens*[23,24] and of the metabolic behavior of *Asp. niger* growing in glucose media[25]. These facts may probably be based upon the inhibitory effect of arsenite for the deamination of alanine and further metabolic process of pyruvic acid such as decarboxylation. The curve indicating the amount of pyruvic acid in the medium added with arsenite showed a similar tendency with that of ammonia produced (Fig. 2, Fig. 3), although the amount of pyruvic acid detected was somewhat lower than that calculated on the basis of the amount of ammonia liberated, which will show the possibility of utilization of some parts of pyruvic acid formed even in the presence of 5 ml of M/20 arsenite. The other characteristic of alanine is that it is preferentially utilized by the present fungus, especially in a relatively earlier-stage of growth, to proper carbon source scuh as glucose (Table I), as it was recognized with glutamic acid by Tanaka[9]. The consumption of alanine was observed to be far superior to that of glucose during the first ten days of growth, thereafter, followed by a gradual increase of the consumption of glucose, though it did not reach the consumption of alanine. After the twentieth day of incubation, a remarkable increase of consumption of glucose, compared to the relatively slower increase of consumption of alanine, was recognized. This characteristic behavior of *P. oryzae* towards both gultamic acid and alanine may present one hopeful step towards the investigation of the biochemical relationship existing between this fungus and the host plant.

SUMMARY

(1) *Piricularia oryzae* is able to utilize DL-norleucine through its conversion to α-keto-caproic acid, although, the natural occurrence of this amino acid still remains questionable. This keto acid is identified as its 2,4-dinitrophenylhydrazone.

(2) In the utilization of DL-alanine by *P. oryzae* some parts of this amino acid were utilized in the form of amino acid itself and other parts via prior conversion to pyruvic acid, but the accumulation of the present

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[23] M. Kunimi and S. Hirano, *Medicine and Biology Japan*, **31**, 197, (1954).

[24] S. Hirano, *Medicine and Biology Japan*, **32**, 262, (1954).

[25] M. I. D. Chughtai, A. A. Pearce and T. K. Walker, *Biochem. J.*, **47**, 135, (1950).

keto acid was observed only in the presence of arsenite, as was already recognized with other several microorganisms.

(3) It was established that alanine is preferentially utilized to a proper carbon source such as glucose, as it was recognized with glutamic acid. This may present a probable step towards solving the problem of biochemical mechanism of invasion of this fungus and its further growth in the host plant.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 2, p. 108~116, 1958]

Biochemical Studies of *Piricularia oryzae*

Part VI. Behavior towards DL-α-Aminobutyric Acid and DL-Isoleucine: Formation of α-keto-β-Methylvaleric Acid from either Amino Acid.

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Received November 13, 1957

When DL-α-aminobutyric acid was used as a supplement in successive studies on the behavior of *Piricularia oryzae* towards amino acids, a keto acid differing from the α-keto analogue of this amino acid was isolated as its 2,4-dinitrophenylhydrazone upon which this hydrazone was found to be identical with the 2,4-dinitrophenylhydrazone of α-keto-β-methylvaleric acid which was formed from DL-isoleucine in another experiment. This finding indicates the formation of a six carbon compound from four carbon amino acid. The probable role of α-aminobutyric acid as an intermediate in the biosynthetic pathway of isoleucine has been discussed by several investigators, mainly based on the biochemically deficient mutant strain method and more recently on the isotopic competition method. Our present data seem to demonstrate direct evidence supporting the biosynthetic route of isoleucine in which α-aminobutyric acid or its α-keto analogue is included as a precursor of isoleucine. In addition, the presence of a close metabolic relationship between α-aminobutyric acid and isoleucine may be suggested from the fact that pyruvic acid and α-ketoglutaric acid were recognized together in respective experiment in which either α-aminobutyric acid or isoleucine was used as the supplement in the presence of arsenite.

In the successive study of the behavior of *Piricularia oryzae* towards amino acids, DL-α-aminobutyric acid was employed as the amino acid to be investigated. In this experiment a keto acid differing from the α-keto analogue of this amino acid was isolated as its 2,4-dinitrophenylhydrazone from the culture medium, which was assumed to be the 2,4-dinitrophenylhydrazone of a six carbon keto acid, especially of α-keto analogue of isoleucine, from some experimental data[1)]. In order to obtain the sample comparable with the 2,4-dinitrophenylhydrazone from α-aminobutyric acid just mentioned, and also in order to find the behavior towards isoleucine, a similar incubation experiment as previous[2)]

[1)] T. Shimomura and Y. Nakamura, This Bulletin, **19**, 178, (1955).

[2)] T. Shimomura and Y. Nakamura, part V of this series.

was undertaken with DL-isoleucine, and the α-keto acid corresponding to this amino acid, i.e., α-keto-β-methylvaleric acid, was successfully separated and identified as its 2,4-dinitrophenylhydrazone. This hydrazone was proved to be identical with that derived from DL-α-aminobutyric acid. This fact indicates the formation of a six carbon compound of isoleucine carbon skeleton from four carbon amino acid. Much interest was devoted to the biosynthesis of isoleucine and valine as a result of Bonner et al.[3,4] describing a *Neurospora* mutant requiring both of these amino acids. In earlier reports, it was demonstrated that these two amino acids might origin in common four carbon compounds metabolically related to threonine[5,6]. More recently, however, independence of the biosynthetic pathway of two amino acids has been presented by Adelberg et al.[7,8,9] and Hirsch et al.[10] and, in addition the experimental results by Strassman et al.[11,12] and Abelson[13] seem to be favorable to such an independent biosynthetic route. On the basis of nutritional experiments with induced mutant strains of microorganisms and isotopic competition experiments, four carbon amino acids such as homoserine, threonine and α-aminobutyric acid have been presented in the biosynthetic pathway of isoleucine by several investigators[5,6,13,14,15,16]. Practically, α,β-dihydroxy-β-methylvaleric acid was separated and indentified as the precursor of isoleucine from the culture medium of *Neurospora* mutant fed with either acetate[17] or threonine[8,9], and Wagner[18] succeeded in obtaining α-keto-β-methylvaleric acid from the culture filtrate of *Neurospora* mutant fed with threonine. In spite of the nutritional significance of α-aminobutyric acid as an intermediate in the biosynthetic route of isoleucine, the direct separation of isoleucine carbon chain compound, as was observed with threonine, derivable from this amino acid is yet unknown. The present report deals with the formation of α-keto-β-methylvaleric acid by *P. oryzae* from DL-α-aminobutyric acid as well as from DL-isoleucine and furthermore, the close metabolic relationship between these two amino acids, which is suggested by the fact that both pyruvic acid and α-ketoglutaric acid are recognized together in the presence of arsenite in respective experiment added with either α-aminobutyric acid or isoleucine as the supplement.

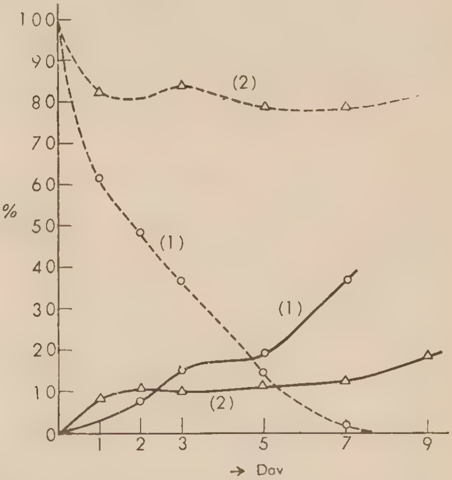

FIG. 1. Ammonia- and Amino-Nitrogen from DL-Isoleucine by *P. oryzae*.

(1), DL-isoleucine only; (2), DL-isoleucine + arsenite. Full line expresses ammonia-nitrogen and dotted, amino-nitrogen. Values are expressed as percentage to theoretical value of nitrogen of isoleucine supplemented.

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[3] David Bonner, E. L. Tatum and G. W. Beadle, *Arch. Biochem.*, **3**, 71, (1943-44).;

[4] David Bonner, *J. Biol. Chem.*, **166**, 545, (1946).

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[6] H. Edwin Umbarger and Edward A. Adelberg, *J. Biol. Chem.*, **192**, 883, (1952).

[7] Edward A. Adelberg, *J. Amer. Chem. Soc.*, **76**, 4241, (1954).

[8] Edward A. Adelberg, Carroll A. Coughlin and R. W. Barrat, *J. Biol. Chem.*, **216**, 425, (1955).

[9] Edward A. Adelberg, *J. Biol. Chem.*, **216**, 431, (1955).

[10] Marie Louise Hirsch and Georges N. Cohen, *Chem. Abstr.*, **49**, 6378, (1955).

[11] M. Strassman, A. J. Thomas and S. Weinhouse, *J. Amer. Chem. Soc.*, **75**, 5135, (1953).

[12] M. Strassman, A. J. Thomas, L. A. Locke and S. Weinhouse, *J. Amer. Chem. Soc.*, **76**, 4241, (1954).

[13] Philip Abelson, *J. Biol. Chem.*, **206**, 335, (1954).

[14] H. Edwin Umbarger and J. Howard Mueller, *J. Biol. Chem.*, **189**, 277, (1951).

[15] Edward A. Adelberg, *J. Bact.*, **61**, 365, (1951).

[16] H. Amos and G. N. Cohen, *Biochem. J.*, **57**, 338, (1954).

[17] Edward A. Adelberg, David M. Bonner and E. L. Tatum, *J. Biol. Chem.*, **190**, 837, (1951).

[18] R. P. Wagner and Arloa Bergquist, *J. Biol. Chem.*, **216**, 251, (1955).

EXPERIMENTAL AND RESULTS

Incubation—The general procedure was similar to that described in Part V of this series: two mycelial felts grown in a normal medium[1] for 17 days were transferred to a flask containing 60 ml of the second culture medium which consisted of 40 ml of M/15 phosphate buffer solution (pH 6.9), 20 ml of water and 100 mg of DL-isoleucine or DL-α-aminobutyric acid and further incubation was made at 27~8°. In the inhibition experiment, 5 ml of M/20 potassium arsenite was added in place of 5 ml of water. At appropriate intervals, ammonia- and amino-nitrogen contents were determined by usual methods. The same determinations were applied to the filtrate containing 144 mg of sodium formate besides 100 mg of aminobutyric acid. The results are shown in Fig. 1, Fig. 2 and Fig. 3.

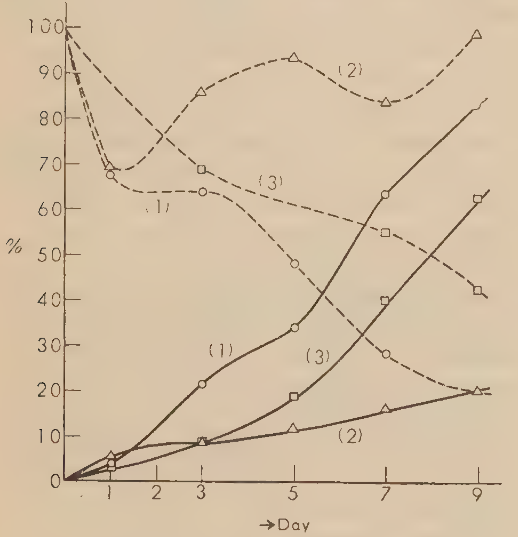

FIG. 2. Ammonia- and Amino-Nitrogen from DL-α-Aminobutyric Acid by *P. oryzae* (I).

(1), DL-α-aminobutyric acid only; (2), aminobutyric acid + arsenite; (3), aminobutyric acid + formate. Expressions are the same as in Fig. 1.

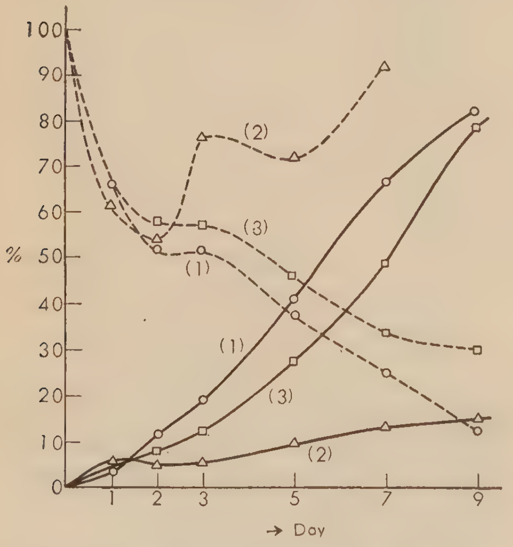

FIG. 3. Ammonia- and Amino-Nitrogen from DL-α-Aminobutyric acid by *P. oryzae* (II).

All expressions are the same as in Fig. 2.

Separation and Identification of Keto Acids—On basis of the results given in previous papers[1,2] the separation of keto acids was carried out mainly with the culture filtrate added with arsenite. In this case, the incubation mixture consisted of 35 ml of M/15 phosphate buffer solution (pH 6.9), 5 ml of M/20 potassium arsenite and 50 mg of DL-isoleucine per flask. On the fifth day of incubation at 27~8°, the mycelial felts were removed by filtration and 180 ml of the filtrate was treated in the same manner as described in previous report[1,2] (basic ether extraction-8 hours; acidic ether extraction-20 hours). The crystalline mass of the 2,4-dinitrophenylhydrazone obtained was divided into two fractions, that is, readily soluble in cold 94 per cent alcohol and slightly soluble in the same alcohol. Upon being recrystallized with hot alcohol, the latter fraction gave a yellow hexagonal plate, which melted at 213~4° and showed no depression in the determination of a mixed melting point with authentic pyruvic acid. In the mixed paper chromatography in which *n*-butanol saturated with water was used as the solvent, the present hydrazone of $R_F=0.24$ corresponded to anti-type of 2,4-dinitrophenylhydrazone of pyruvic acid. In some cases, a dilute spot of syn-type of pyruvic acid 2,4-dinitrophenylhydrazone ($R_F=0.46$) was also observed besides the clear spot of anti-type[19]. These data indicate that the present hydrazone is that of pyruvic acid. Yield, Ca. 7 mg. Careful addition of water to the former fraction gave rise to a mixture of long needles and fine crystals, which showed two clear spots on paper strip $R_F=0.24$ and 0.85, respectively. From this it seems that the lower spot is related to the 2,4-dinitrophenylhydrazone of pyruvic acid and the upper spot to 2,4-dinitrophenylhydrazone of α-keto analogue of isoleucine. After being kept at 0° overnight, the crystalline mass was separated by filtration. Separation of this mixture into respective component was carried out by cellulose column chromatography[20]: cellulose powder (Toyo Roshi Kaisha, Ltd.) suspended in *n*-butanol saturated with water was carefully poured onto a glass funnel (1×25 cm) to the height of 14 cm without suction and was allowed to stand overnight. The solution of 2,4-dinitrophenylhydrazone mixture just mentioned, in a minimal amount of water-saturated *n*-butanol and alcohol was dropped into the column and successive development with the same solvent was carried out at the flow rate of about 5 ml per hour. Three yellow bands could be observed on the cellulose column. The effluent of each band was reduced to dryness in vacuo at room temperature and the residual mass was treated with alcohol containing water. From the third effluent a yellow plate (m.p., 216°) was obtained in a small quantity, which was identified to be 2,4-dinitrophenylhydrazone of pyruvic acid in the same manner as that just described. From the second, a small quantity of amorphous substance was separated, but not any crystalline substance, except a trace quantity of pyruvic acid 2,4-dinitrophenylhydrazone contaminated. The repeated recrystallization of the first effluent gave a yellow crystal of long needles, melting at 164~6° (Found: C, 46.44; H, 3.86; N, 18.24. Calcd. for $C_{12}H_{14}O_6N_4$: C, 46.45; H, 4.51; N, 18.07) and also R_F=0.86 in paper chromatography in a *n*-butanol water solvent. Yield, Ca. 0.8 mg. Although these analytical values were in good accordance with those of 2,4-dinitrophenylhydrazone of α-keto analogue of isoleucine, the melting point of the present hydrazone is somewhat below 176°, the melting point of 2,4-dinitrophenylhydrazone of *d*- or *l*-α-keto-β-methylvaleric acid, reported by Meister[21,22]. In this case, however, some attention must be paid to the fact that the melting point of 2,4-dinitrophenylhydrazone of the racemic mixture of this keto acid is 168~9°[21]. The 2,4-dinitrophenylhydrazone of pure *d*-α-keto-β-methylvaleric acid (kindly supplied by Dr. A. Meister) was prepared by the authors and its melting point was confirmed as 172~3°. In order to obtain 2,4-dinitrophenylhydrazone of recemic mixture, the following procedure was carried on according to Meister[21]: the aqueous solution of *d*-α-keto-β-methylvaleric acid was adjusted to pH, 9.5 with 0.1 N sodium hydroxide and kept at room temperature for about half an hour, followed by the back-adjustment to acidic condition with 0.1 N hydrochloric acid. The 2,4-dinitrophenylhydrazone from this solution melted at 164~6° and was proved to be identical with the 2,4-dinitrophenylhydrazone obtained from the first effluent, by paper chromatography and mixed melting point determination. These data show the formation of racemic α-keto analogue of isoleucine. When the yellow filtrate from which the mixture of 2,4-dinitrophenylhydrazones of pyruvic acid and α-keto-β-methylvaleric acid was removed, was treated with a further addition of water, a pale yellow crystal was separated in a slight amount. Upon being recrystallized with water, it melted at 217~8° and showed R_F=0.07 in paper chromatography in a *n*-butanol water solvent. This hydrazone was identified to be that of α-ketoglutaric acid by the mixed paper chromatography and mixed melting point determination with an authentic sample. But this hydrazone was only detected in rare cases. The above procedure is summerized briefly in the following diagram:

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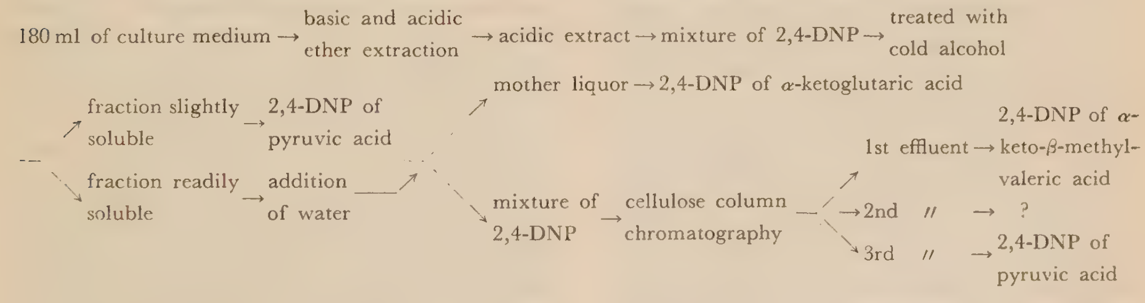
[19] T. Moriwaki, M. Katsuki and S. Tanaka, *J. Chem. Soc. Japan*, **76**, 1367, (1955).


180 ml of culture medium →
basic and acidic ether extraction
→ acidic extract → mixture of 2,4-DNP →
treated with cold alcohol
fraction slightly soluble → 2,4-DNP of pyruvic acid
fraction readily soluble → addition of water
mother liquor → 2,4-DNP of α-ketoglutaric acid
mixture of 2,4-DNP → cellulose column chromatography
1st effluent → 2,4-DNP of α-keto-β-methylvaleric acid
→ 2nd // → ?
3rd // → 2,4-DNP of pyruvic acid

The isolation and identification of keto acids derivable from DL-α-aminobutyric acid was conducted with the culture filtrate of the same incubation conditions as stated above. Many experiments were conducted in the presence or absence of arsenite. In the absence of arsenite, the isolation of 2,4-dinitrophenylhydrazone of six carbon keto acid was comparatively easy, probably owing to the absence of other keto acids and other unknown carbonyl compounds, though its yield was unsatisfactory. On the other hand, arsenite stimulated the relative accumulation of pyruvic acid, α-ketoglutaric acid and others as well as six carbon keto acid, but the separation procedure was pretty difficult. Some of these experiments are described as follows: a portion of 230 ml of the filtrate without arsenite on the fourth day of incubation was treated in the same way as just described and a yellow mass of 2,4-dinitrophenylhydrazones was dissolved in cold 94 per cent ethanol and insoluble dark red substances were discarded. The yellow solution was added dropwisely with water until slight turbidity was observed. After being warmed in the water bath, the solution was allowed to stand still. A rod-shape-like crystal separated and melted at about 150°. Recrystallization in the same manner resulted in a pure yellow crystal, m.p., 165∼7°[*]; R_F=0.88∼0.91 in butanol-ammonia-water solvent and R_F=0.85 in butanol-water solvent (Found: C,46.86; H, 5.02. Calcd. for $C_{12}H_{14}O_6N_4$: C, 46.45; H,4.51). Yield, Ca. 0.8 mg. The present hydrazone was proved to be identical with that of racemic mixture of α-keto analogue of isoleucine by paper chromatography and mixed melting point determination. In the presence of arsenite, 72 ml of a seven-day old culture filtrate was treated in a similar manner as in the case of isoleucine and the mixture of 2,4-dinitrophenylhydrazones gave two clear spots (R_F=0.04 and 0.22) and one streaming spot with a larger R_F on paper strip (*n*-butanol-water solvent; ascending method). This mixture was subjected to the column chromatography just mentioned. On the cellulose column four bands appeared, designated as No. 1, No. 2, No. 3 and No. 4 starting from the lowest band, but the separation of No. 1 and No. 2 was not so distinct between each other. Similar treatment as in the case of isoleucine was applied to each effluent. From No. 4, a pale yellow plate, m.p., 217∼8°, was obtained and was identified to be 2,4-dinitrophenylhydrazone of α-ketoglutaric acid by mixed melting point determination and paper chromatography (R_F=0.08). Yield, Ca. 0.4 mg. The yellow crystal from No. 3 was identified to be 2,4-dinitrophenylhydrazone of pyruvic acid by the same methods. Yield, Ca. 1.5 mg. The mixture of No. 1 and No. 2 gave a needle-like crystal, which did not show sharp melting point but softened at about 120∼30° and partially liquefied. The application of paper chromatographic technique indicated that this crystalline hydrazone consisted of three components showing R_F=0.44, 0.62 and 0.82, respectively. The yellow color of the two lower-spots was very weak and also, the separation was not clear between each other. From the color-intensity and area of each spot it was assumed that a greater part of this hydrazone mixture was occupied by the hydrazone of R_F=0.82 which was recognized to be identical with 2,4-dinitrophenylhydrazon of isoleucine keto analogue by parallel paper chromatography. The other two components are not known at present, but the hydrazone of R_F= 0.44 may be that of α-ketobutyric acid[23], the formation of which would possibly be deduced from several experiments conducted so far by the authors and the hydrazone of R_F=0.62 might be that of valine keto analogue[18]. In addition, 2,4-dinitrophenylhydrazone of six carbon keto acid was chromatographically separated from the 2,4-dinitrophenylhydrazones which were prepared directly by the addition of the reagent to the original filtrate and thus, about 1 mg of 2,4-dinitrophenylhydrazone (m.p., 166°) of isoleucine keto analogue was obtained from 40 ml of a four-day old culture medium supplemented with formate, and a small quantity of this hydrazone (m.p. 165∼6°), from 40 ml of the four-day old filtrate added with arsenite together with a minimal amount, in general, from the filtrate on the fifth day added with aminobutyric acid only. In most cases, yellow bands of keto acids hydrazones other than the 2,4-dinitrophenylhydrazone of isoleucine keto analogue were not observed on the column, except an unidentified faint orange hydrazone. This fact may be due to the presence of keto acids, especially pyruvic acid, in a certain combined form. This problem is under investigation.

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[20] K. Satake and K. Narita, Jikken-Kagaku-Koza, Maruzen, Tokyo, 1956, 2-II, P. 259.

[21] Alton Meister, *J. Biol. Chem.*, **190**, 269, (1951).

[22] Alton Meister and Patricia A. Abendschein, *Analyt. Chem.*, **28**, 171, (1956).

In general, the isolation of six carbon keto acid as its 2,4-dinitrophenylhydrazone from the medium supplemented with aminobutyric acid was far more difficult in the procedure, thus resulting more unsatisfactory in yield than from the medium containing isoleucine as the supplement. This may suggest the metabolic complexity and variety involved in the conversion process of aminobutyric acid to α-keto analogue of isoleucine. In addition, some attention should be paid to the fact that the employment of the successive culture of *P. oryzae* on agar plate brought the gradual decrease of the yield of this keto acid

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[*] The melting point of 153∼4° reported in previous paper(1) was an uncorrect value due to technical carelessness. On further determination of the melting point of the same preparation with another precise thermometer, a constant m.p. of 165∼7° was observed.

[23] K. Satake and K. Narita, Jikken-Kagaku-Koza, Maruzen, Tokyo, 1956, 2-II, P. 226.

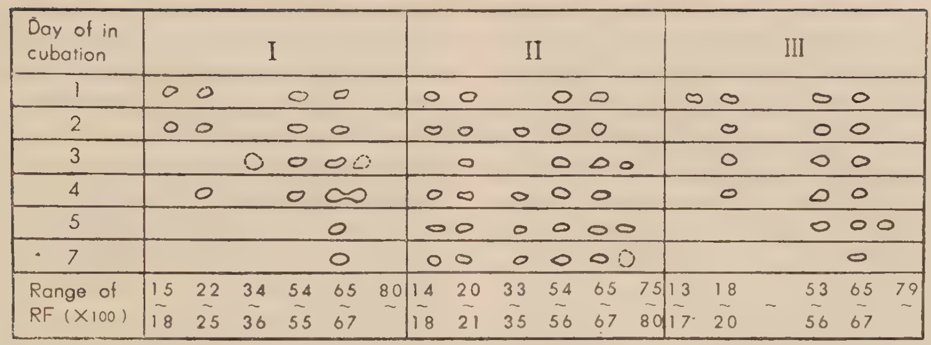

FIG. 4. Detection of Amino Acids in the Culture Filtrate by Paper Chromatography.

Experimental conditions, see text. I, indicates the case of aminobutyric acid only; II, the case of aminobutyric acid+arsenite; III, the case of aminobutyric acid+formate.

hydrazone.

Paper Chromatography of Amino Acids—Detection of amino acids in the culture solution was made by paper chromatography (ascending method, phenol-water solvent and room temperature). The condition of incubation was identical as described above. Several experiments were conducted repeatedly and the similarity in the appearance and disappearance of ninhydrin-positive spots was observed in either case of three incubation conditions, that is, aminobutyric acid only, the same amino acid plus arsenite and the same amino acid plus formate, although small unconformity was unavoidable owing to the use of intact mycelial felt. An example of these experiments is diagrammatically illustrated in Fig. 4.

The culture filtrate was extracted with ether at pH 8.5 and successively at pH 4.0 for five hours respectively and the remaining aqueous solution was reduced to dryness in vacuo. The extract of the residue with 70 per cent alcohol was evaporated and dissolved in a small amount of water. The application of paper chromatography to this aqueous solution indicate all other spots on paper strip except spot $R_F=0.15\sim0.18$. In parallel chromatography employing pure amino acids, spot $R_F=0.18\sim0.25$ corresponded to glutamic acid, spot $R_F=0.53\sim0.56$ to alanine, spot $R_F=0.65\sim0.67$ to aminobutyric acid and spot $R_F=0.75\sim0.80$ to valine. The spot of $R_F=0.33\sim0.35$ may be that of serine, but it remains uncertain. The spot indicating the minimum R_F is at present still unknown.

DISCUSSION

The nutritional significance of isoleucine towards microorganisms has been studied in recent years and it has been chiefly considered on the basis of enzymic experiments that the formation of α-keto acid by way of deamination would proceed as one of its metabolic changes. However, the formation of the α-keto analogue of isoleucine by intact microorganisms has seldom been observed. In the experiment using an *Escherichia* mutant, evidence by paper chromatography indicating the formation of α-keto analogue of isoleucine in the culture medium supplemented with DL-isoleucine was reported by Umbarger et al.[24], but the identification of this keto acid has so far not been presented. Recently, Wagner and Bergquist[18] reported on the formation of this keto acid (racemic) not from isoleucine but threonine, using an induced strain of *Neurospora*. In our present experiment, the formation of racemic α-keto acid analogue from DL-isoleucine by *P. oryzae*, as was observed with other various amino acids[1,2,25], was recognized by the separation and identification of its 2,4-dinitrophenylhydrazone. These data indicate that the present fungus has the ability to utilize isoleucine as a carbon source as well as a nitrogen source. It was supposed in previous papers[1,2] that this fungus was able to deaminate either form of amino acids used, D or L, in accordance with the amount of ammonia produced. The present identification of racemic mixture of isoleucine keto analogue, especially in the original filtrate untreated in any way, seems to give additional evidence supporting such interpretation. In either incubation added with arsenite or without arsenite, the change of the amount of ammonia- and amino-nitrogen in the medium showed a similar tendency as these of previous reports, and in the present incubation experiment without arsenite, additional evidence was obtained that some parts of isoleucine added were absorbed and utilized in the form of amino acid itself, that is, 36.3 per cent of isoleucine supplemented on the second day of incubation and 66.2 per cent on the fifth day, for instance. It is noteworthy that it was only in the presence of arsenite, that pyruvic acid was nearly always detectable and also rarely in case of α-ketoglutaric acid. These results can be interpreted to mean that either keto acid is formed from isoleucine or isoleucine indirectly stimulates the endogenous production of keto acids. If the former relationship is to be held, this fact will present a new problem in the study of the metabolic fate of isoleucine. In addition, the application of paper chromatography to the culture filtrate containing arsenite (two-day and four-day old) showed spots corresponding to alanine, but rarely to glutamic acid, besides isoleucine. The experimental results that pyruvic acid derivable from alanine was detected only in the presence of arsenite[2,26] may suggest that pyruvic acid recognized in the present incubation is possibly derivable from alanine. Coon and associates[27,28] found the formation of 2-methylbutyrate and propionate as the intermediate in the metabolism of isoleucine and described the probable formation of pyruvate from propionate. Our present results appear to be favorable for such hypothesis, at least in concern of the formation of pyruvate. At present it is uncertain whether isoleucine might be directly convertible to these amino acids or not, but the independence of the amino acids upon autolysis will be presumed from the decrease of concentration or their disappearance in the later-stage of incubation and any amino acid except these two amino acids (alanine and glutamic acid) being undetectable.

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[24] H. Edwin Umbarger and Boris Magasanik, *J. Biol. Chem.*, **189**, 287, (1951).

[25] Y. Nakamura and T. Shimomura, *J. Agr. Chem. Soc. Japan*, **28**, 694, (1953).

When DL-α-aminobutyric acid was used as the supplement, a keto acid other than the α-keto analogue of this amino acid was separated as its 2,4-dinitrophenylhydrazone in either incubation condition, which was identified to be that of *dl*-α-keto-β-methylvaleric acid. This fact indicates the formation of branched six carbon compound of isoleucine carbon skeleton from four carbon amino acid. In addition to this, the almost constant formation of pyruvic acid and rare formation of α-keto-glutaric acid were observed in the presence of arsenite. On the basis of nutritional experiments conducted with induced mutant strains of microorganisms such as *Neurospora* or *E. coli* and isotopic competition experiments, four carbon amino acids such as homoserine, threonine and α-aminobutyric acid were presented as the intermediate in the biosynthetic pathway of isoleucine[5,6,13,14,15,16]. Practically, Adelberg et al.[5,8,9,17] successfully isolated α,β-dihydroxy-β-methylvaleric acid from the culture medium of *Neurospora* strain fed with threonine[8,9] or acetate[5,17], besides α,β-dihydroxyisovaleric acid, the presumable valine precursor and also recognized the effectiveness of aminobutyric acid in the accumulation of α,β-dihydroxy-β-methylvaleric acid. Recently, Wagner and Bergquist[18] identified acetaldehyde, pyruvic acid, α-ketoisovaleric acid and α-keto-β-methylvaleric acid in the culture medium of *Neurospora* strain added with threonine. In spite of such experiments as this, the direct separation of a compound having isoleucine carbon chain from α-aminobutyric acid has not yet been

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[26] M. Kunimi and S. Hirano, *Medicine and Biology Japan*, **31**, 197, (1954).

[27] Minor J. Coon and Norman S. B. Abrahamsen, *J. Biol. Chem.*, **195**, 805, (1952).

[28] Minor J. Coon, Norman S. B. Abrahamsen and Grace S. Greene, *J. Biol. Chem.*, **199**, 75, (1952).

recognized, except findings of the nutritional effectiveness of this amino acid. The present identification of α-keto analogue of isoleucine will present direct evidence indicating that α-aminobutyric acid can be included in the biosynthetic route of isoleucine. At the same time, this fact suggests that such biosynthetic route of isoleucine will probably prevail, at least, in microorganisms. In the present experiment α-ketobutyric acid was not separated unexpectedly, but it would be rather possible to regard that the deamination of aminobutyric acid would proceed in the same way as already observed with other several amino acids, although in the present case this keto acid was not detected distinctly. Indeed, in some cases, a spot presumably corresponding to α-ketobutyric acid was observed in paper chromatography besides α-keto-β-methylvaleric acid. Wagner et al.[18], described that six carbon compound formed by aldol condensation of acetaldehyde and α-ketobutyric acid which was derivable from threonine[29] was followed by a pinacol type rearrangement to give a dihydroxy precursor of isoleucine, which was then converted to α-keto acid precursor, i.e., α-keto-β-methylvaleric acid, as already postulated by Adelberg[7,9] and Strassman[12], and also that pyruvic acid derivable from threonine had relation to the formation of valine carbon chain via a similar reaction. However, Adelberg[8,9] reported that the independent biosynthetic route of solieucine upon that of valine was based on the use of C^{14}-labeled compound. Moreover, Wagner et al. postulated that pyruvic acid found in their experiment might be derived from serine formed from glycine[30,31] resulting from the cleavage of threonine to acetaldehyde and glycine[32]. In our present experiment conducted in the presence of arsenite, pyruvic acid was nearly always identified and, by paper chromatography the appearance of alanine and presumable serine was rarely observed in the culture medium. These results make it possible to consider that pyruvic acid may be derivable from alanine as it has been discussed in the case of isoleucine. The interconversion of alanine and serine[33] and the metabolic similarity of both amino acids in the formation of isoleucine carbon chain[34] seem to be favorable for discussing elucidation of the derivation of pyruvic acid from alanine. In discussion of the formation of pyruvic acid from threonine, Wagner et al. considered the transfer of one carbon unit according to Sakami, as just described. *P. oryzae* was incubated in the medium supplemented with both aminobutyric acid and sodium formate and the possibility of utilization of the formate was recognized by the smaller formation of ammonia and a smaller consumption of amino acid than in the case added with aminobutyric acid only (Fig. 2 and Fig. 3). Furthermore, it was found that this fungus could utilize 75.2 per cent of sodium formate on the third day and 92.9 per cent on the seventh day in the incubation experiment in which 75 mg of glycine and 100 mg of formate were used as the supplement in the absence of arsenite[35]. Thus, pyruvic acid which was found in the medium—at least, certain parts of this acid—may be formed via such metabolic changes, although neither glycine nor acetaldehyde was detected in the present experiment.

The spot of $R_F=0.75\sim0.80$, which was detected in a relatively later-stage of incubation added with either arsenite or formate, was in all likelihood, assumed to be correspondent to valine, and in most cases it disappeared again, thereafter. This may be interpreted as the additional evidence of the role of aminobutyric acid as a valine pre-

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29) Charles Yanofsky and José L. Reissig, *J. Biol. Chem.*, **202**, 567, (1953).

30) Warwick Sakami, *J. Biol. Chem.*, **176**, 995, (1948).

31) Philip Siekevitz and David M. Greenberg, *J. Biol. Chem.*, **180**, 845, (1949).

32) G. Ya. Vilenkina, *Chem. Abstr.*, **44**, 2582, (1950); **46**, 10227, (1952).

33) Oliver G. Lien, Jr., and David M. Greenberg, *J. Biol. Chem.*, **195**, 637, (1952).

34) G. Ehrensvärd, L. Reio, E. Saluste and R. Stjernholm, *J. Biol. Chem.*, **189**, 93, (1951).

35) Unpublished.

cursor. However, the independence of the biosynthetic route of valine and isoleucine by Adelberg[8,9] must be kept in mind. The examination of a carboxylic acid, confirmed recently by the authors in the residual ether extract after removal of keto acids, may somewhat be contributed to the above interpretation.

The formation of α-ketoglutaric acid and glutamic acid also is unexplainable at present. It may only be a side-reaction deviating from the main pathway of aminobutyric acid. If the following hypothesis that aminobutyric acid and perhaps threonine would act not only as a donor but also as an acceptor of one carbon fragment, is accepted, as it is known with glycine, two moles of aminobutyric acid would yield alanine (pyruvic acid) and valine and further incorporation of one mole of aminobutyric acid in the first reaction would lead to alanine and isoleucine, although the timely sequence of deamination and transfer of one carbon fragment is out of the range of consideration. The contamination of one carbon fragment in the first reaction might result in the formation of glutamic acid or α-ketoglutaric acid.

The change of amino nitrogen in the medium added with arsenite and aminobutyric acid was indeed distinct from those of other experiments: the re-increase of amino-nitrogen was determined on a three-day old culture and that thereafter. The same incubation experiments were repeated thrice and the same results were obtained (Fig. 2 and Fig. 3). The presumable independence of these results on a simple autolysis will be shown by the transparency of the medium and the constancy of paper chromatographic spots. The production of endogenous amino acids or the re-amination of keto acids may possibly be taken into consideration, but at present, no decisive data are available.

At any rate, the presence of a close metabolic relationship between aminobutyric acid and isoleucine is suggested by the isolation of the same keto acids from either amino acid used as the supplement.

SUMMARY

(1) *Piricularia oryzae* is able to utilize DL-isoleucine as a carbon source through its conversion to α-keto analogue, i.e., α-keto-β-methylvaleric acid. This keto acid is identified as its 2,4-dinitrophenylhydrazone (racemic mixture).

(2) In the incubation of *Piricularia oryzae* in the medium supplemented with DL-α-aminobutyric acid, the identification of α-ketobutyric acid was unsuccessful, but the formation of α-keto-β-methylvaleric acid was recognized by the separation of its 2,4-dinitrophenylhydrazone (racemic mixture). This fact presents direct evidence supporting the nutritional significance of aminobutyric acid as an intermediate in the biosynthetic pathway of isoleucine, in addition to the suggestive prevalency of such biosynthetic route, at least, in microorganisms.

(3) The close metabolic relationship between isoleucine and α-aminobutyric acid is suggested by the separation and identification of pyruvic acid, α-ketoglutaric acid and α-keto-β-methylvaleric acid as 2,4-dinitrophenylhydrazone in either incubation added with isoleucine or α-aminobutyric acid in the presence of arsenite.

(4) The availability of formate to this fungus is indicated.

(5) The possible mechanism of the formation of these three keto acids is discussed, taking into account the paper chromatographic evidence obtained with the culture medium.

Acknowledgements. The authors wish to express their hearty thanks to Dr. A. Meister, Prof. of Biochemistry, Tufts University, School of Medicine, Boston, for his kind gift of *d*-α-keto-β-methylvaleric acid and his helpful advice, and to Dr. Y. Otani, Ass. Prof. of Hokkaido University, for his kindly supplying the blast fungus.

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Hydrolysis Rate of Soluble Glucose-Polymer in Dilute Sulfuric Acid

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The purpose of this study is to find the optimum condition of post-hydrolysis in the wood saccharification process with concentrated sulfuric acid. The hydrolysis of the glucose-polymer which is formed by the degradation of cellulose dissolved in concentrated sulfuric acid and is soluble in dilute sulfuric acid has been measured. The hydrolysis of soluble glucose-polymer is of the first-order in sulfuric acid concentrations ranging from 5 to 40% at temperatures within the range of 87 to 130°C. The reaction constant is expressed by the following empirical equation as a function of the acidity function of sulfuric acid (C) and temperature (T°K):

$$k=1.61\times 10^{15}\exp\left[-1.46C-\{28400/(RT)\}\right].$$

INTRODUCTION

The wood saccharification process with strong sulfuric acid consists of three principal stages. Firstly, the hemicellulose of the raw material is removed by the action of hot dilute sulfuric acid. This reaction is called the pre-hydrolysis. Secondly, the residue of of the pre-hydrolysis which mostly consists of cellulose and lignin is treated with concentrated sulfuric acid. In this process which is called the main-hydrolysis, cellulose is converted into glucose-polymers that have low degrees of polymerization. These polymers are soluble in dilute sulfuric acid and are readily hydrolyzed. The concentrated acid, however, is incapable of converting these polymers into glucose-monomers. Thirdly, the glucose-polymers in the main-hydrolyzate are converted to the glucose-monomers through the dilute acid hydrolysis called the post-hydrolysis.

In spite of the important role of post-hydrolysis in the wood saccharification with concentrated acid, available information on this subject is scanty. Therefore, in order to find the optimum condition of the post-hydrolysis, information on the hydrolysis rate of soluble glucose-polymer in dilute acid is necessary.

The previous works carried out by Kobayashi and his co-workers involved the kinetics of pre-hydrolysis1) and the main-hydrolysis2), drying of the pre-hydrolysis residue3), and the relation of some conditions of main-hydrolysis to sugar hield4). The present paper deals with the kinetics of hydrolysis of soluble glucose-polymers in the presence of sulfuric acid in concentrations ranging from 5 to 40 percent at temperatures from 87° C to 130° C over the average degrees of polymerization (av. D.P.) ranging from 3 to 13. The hydrolysis rate of soluble glucose-polymers could be expressed as a function of sulfuric acid concentration and temperature and that function was independent from the av. D.P. of soluble glucose-polymer.

This paper constitutes the seventh of a series of articles dealing with the wood saccharification process with strong sulfuric acid1~6).

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1) T. Kobayashi and Y. Sakai, This Bulletin, **20**, 1 (1956).

2) T. Kobayashi, *Mokuzaitoka Shingikai Hokoku* (Report of the Wood Saccharification Discussion Committee), **No. 1**, 27 (1952).

3) T. Kobayashi, K. Mitachi and K. Tsuyuzaki, *ibid.*, **No. 5**, 35 (1956).

4) T. Kobayashi and T. Ito, *ibid.*, **No. 4**, 5 (1954).

5) T. Kobayashi and Y. Sakai, *ibid.*, **No. 2**, 55 (1953).

6) T. Kobayashi, T. Hirose and K. Mitachi, *ibid.*, **No. 5**, 49 (1956).

TABLE I

PREPARATION OF SOLUBLE GLUCOSE-POLYMERS

Preparation of Glucose-Polymers				Av. D.P. of Soluble Glucose-Polymers	Hydrolysis	
H_2SO_4 Concn. %	$\frac{\text{Cellulose}}{H_2SO_4}$	Time min.	Temp. °C		H_2SO_4 Concn. %	Temp. °C
82	$\frac{1}{2}$	60	35	5.3	40	115
82	$\frac{1}{2}$	40	30	6.8	10	115
82	$\frac{1}{2}$	40	—	4.1	20	130
82	$\frac{1}{2}$	20	—	3.9	20	87
82	$\frac{1}{2}$	20	40	4.4	5	100
77	$\frac{1}{2}$	60	30	8.8	10	87
77	$\frac{1}{2}$	60	30	13.2	5	130
77	$\frac{1}{2}$	60	25	6.7	20	100
77	$\frac{1}{2}$	40	40	4.5	10	100
77	$\frac{1}{2}$	20	30	8.2	5	87
72	$\frac{1}{2}$	60	—	3.0	40	100
72	$\frac{1}{2}$	60	35	6.9	5	115
72	$\frac{1}{2}$	40	30	5.9	20	115
72	$\frac{1}{4}$	20	45	3.5	40	87
68	$\frac{1}{2}$	120	30	3.2	10	130

MATERIALS AND METHOD

Preparation of Soluble Glucose-Polymers. A sulfuric acid solution of the short-chain glucose-polymers was prepared by the following procedure:

A strip of filter paper (qualitative test use, Toyo-Roshi No. 2) ground with a grater was treated with concentrated sulfuric acid in concentrations of 68 to 82%. One-part of cellulose was impregnated with 2 or 4 parts of sulfuric acid (as 100% H_2SO_4) in procelain mortar for 20 to 120 minutes at room temperature. At the end of impregnation, water sufficient to produce in the mixture a sulfuric acid solution in concentrations of 5 to 40% was added, then the mixture produced was centrifuged and the clear supernant was used for the experiment. The av. D.P. of glucose-polymers obtained in dilute sulfuric acid solution ranged from 3 to 13. The conditions of degradation of cellulose in concentrated sulfuric acid and the av. D.P. of the product in dilute sulfuric acid are shown in Table I.

Determination of End Groups and av. D.P. The number of end-groups in the glucose-polymer solution was determined by the modified alkaline hypoiodite method described by Caldwell, Doebbeling and Manian[7].

The total number of basic units in the glucose-polymers solution was determined as follows: Either water or sulfuric acid solution in an amount necessary to result in 8% sulfuric acid in the solution of glucose-polymers was added and the resulted solution was heated at 100°C for 3 hours. After cooling, the solution was neutralized and the end groups were determined.

The av. D.P. was obtained by dividing the total number of basic units in the glucose-polymers solution by the number of end groups existing in the glucose-polymers solution.

Measurement of Hydrolysis Rate. The experiment was carried out in a procedure similar to that of Saeman[8]. Samples (2 ml) of glucose-polymers of the av. D.P. ranging from 3 to 13 in 5, 10 20 and 40% sulfuric acid solutions were sealed in glass tubes and heated at 87, 100, 115 and 130°C for various times in a paraffin bath with a temperature regulator. After they were heated for different times, they were opened and samples in adequate volumes to the analyses of end groups were drawn. The differences between the numbers of end groups of samples and the total numbers of basic units in the sample untreated gave the numbers of surviving bond. The rate of hydrolysis was computed from the straight line, percentage of the bond's surviving *versus* time and the rate of decomposition of glucose-monomers was taken out of consideration, because that of the former was extremely great in comparison with the latter.

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[7] M. L. Caldwell, S. E. Doebbeling and S. H. Manian, *Ind. Eng. Chem., Anal. Ed.*, **8**, 181 (1936)

[8] J.F. Saeman, *Ind. Eng. Chem.*, **37**, 43 (1945).

RESULTS

The extents of hydrolysis of glucose-polymers at different times under various conditions are given in Figs. 1 to 4. The straight lines which were fitted by the least squares method indicate that the reactions were of the first-order. The slope of the straight line multiplied by –2.303 gave the first-order reaction constant. The first-order reaction constants under various conditions are summerized in Table II.

DISCUSSION AND CONCLUSION

Effect of Av. D.P. to Hydrolysis Rate. Table II gives the first-order reaction constants observed and the initial av. D.P. of glucose-polymers subjected to the measurement of the hydrolysis rates. The data in this table were analyzed by the analysis of covariance[8a] after the missing value was calculated. The

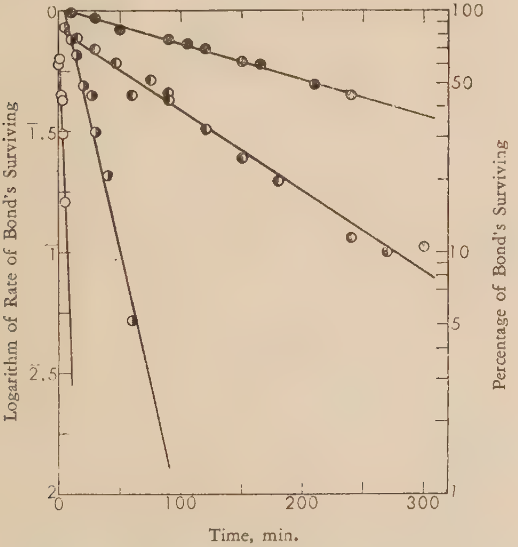

FIG. 1. Hydrolysis of Soluble Glucose Polymers in 5, 10, 20 and 40% Sulfuric Acid at 87°C.

87°C
● 5% Sulfuric Acid ◑ 10% Sulfuric Acid
◑ 20% Sulfuric Acid ○ 40% Sulfuric Acid

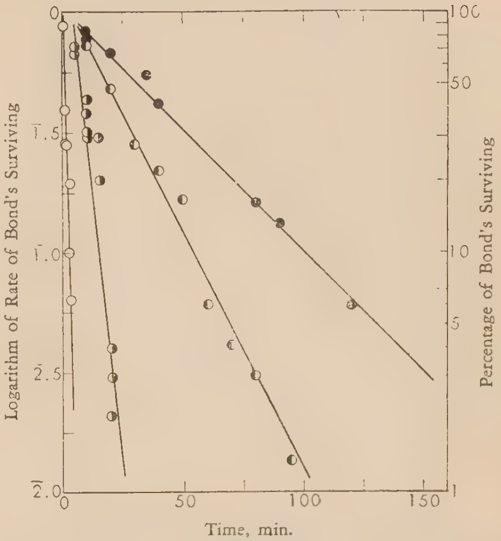

FIG. 2. Hydrolysis of Soluble Glucose-Polymers in 5, 10, 20 and 40% Sulfuric Acid at 100°C.

100°C
● 5% Sulfuric Acid ◐ 10% Sulfuric Acid
◑ 20% Sulfuric Acid ○ 40% Sulfuric Acid

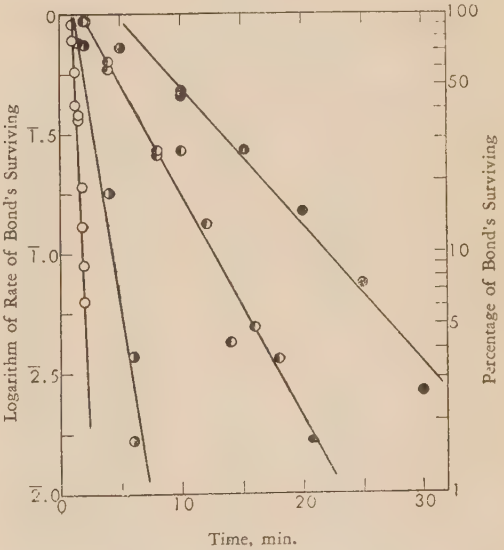

FIG. 3. Hydrolysis of Soluble Glucose-Polymers in 5, 10, 20 and 40% Sulfuric Acid at 115°C.

115°C
● 5% Sulfuric Acid ◐ 10% Sulfuric Acid
◑ 20% Sulfuric Acid ○ 40% Sulfuric Acid

[8a] G. Taguchi, Zikken-keikaku-hô (1), Chemical Society of Japan, 1952.

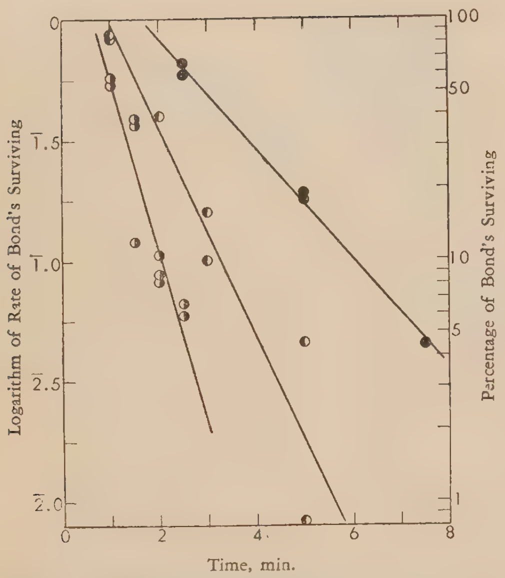

FIG. 4. Hydrolysis of Soluble Glucose-Polymers in 5, 10, 20 and 40% Sulfuric Acid.

130°C
● 5% Sulfuric Acid ◐ 10% Sulfuric Acid
◑ 20% Sulfuric Acid

TABLE II

HYDROLYSIS RATE CONSTANTS OF GLUCOSE-POLYMERS SOLUBLE IN DILUTE SULFURIC ACID

Temp. °C	Strength of Sulfuric Acid		Av. D.P. of Polymers	1-st Order Reaction Constant, *k* (min.$^{-1}$)	
	%	Ho*		Obsd.	Calcd.**
87	5	+0.24	8.2	0.00342	0.00623
	10	−0.16	8.8	0.00772	0.0112
	20	−0.89	3.9	0.0505	0.0323
	40	−2.28	3.5	0.314	0.244
100	5	+0.24	4.4	0.0233	0.0260
	10	−0.16	4.5	0.0452	0.0466
	20	−0.89	6.7	0.209	0.135
	40	−2.24	3.0	1.13	0.981
115	5	+0.24	4.3	0.132	0.109
	10	−0.16	6.8	0.214	0.195
	20	−0.89	5.9	0.750	0.563
	40	−2.28	5.3	2.33	4.26
130	5	+0.24	13.2	0.522	0.454
	10	−0.16	3.2	0.993	0.813
	20	−0.89	4.1	1.62	2.35

* Acidity function[9]
** Calculated from $k=1.61\times10^{15}[-1.46C-\{28400/(RT)\}]$

regression coefficient of the av. D.P. of glucose-polymers on the logarithm of the hydrolysis rate constant is -0.000796. The variances due to regression and about regression are 0.0000469 and 0.334 respectively, hence the regression coefficient is not significant. This result shows that the initial chain length of soluble glucose-polymer has no effect on the rate of its hydorlysis. This signifies that all of the glucose-polymers soluble in dilute sulfuric acid are hydrolyzed at the same velocity under the same condition of temperature and acid concentration, provided their are dissolved.

Hydrolysis Rate as a Function of Temperature and Sulfuric Acid Concentration. According to J. F. Saeman[8], the hydrolysis rate of cellulose and the decomposition rate of glucose can be expressed by the equation:

$$k=H\cdot C^{M}\exp\{-E/(RT)\}$$

or $\log k=\log H+M\cdot\log C-\{E/(2.303\,RT)\}$ (1)

where k is the first-order reaction constant, H a constant, C the percentage concentration of sulfuric acid, M a constant expressing the effect of acid concentration, E the energy of activation, R the molar gas constant, and T the absolute temperature. If the acidity function[9], H_o, is used instead of the logarithm of the percentage concentration of sulfuric acid, log C, equation (1) fits well the present data. Then, equation (1) can be rewritten in the following form:

$$\log k=\log H+(M/2.303)\cdot H_o-\{E/(2.303\,RT)\}$$

or $k=H\exp[M\cdot H_o-\{E/(RT)\}]$. (2)

When the logarithms of the reaction rate constants in Table II are plotted as a function of acidity function, H_o, parallel straight lines are obtained in regular intervals, as shown in Fig. 5. When the logarithms of the reaction rate constant are plotted as a function of reciprocal of the absolute temperature, parallel straight lines are obtained in intervals corresponding to the acidity function, as shown in Fig. 6. These facts prove that equation (2) fits well the present data. The value for M is obtained by multiplying the slope of the straight

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[9] L. P. Hammett, Physical Organic Chemistry, McGraw Hill, 1940, p. 268.

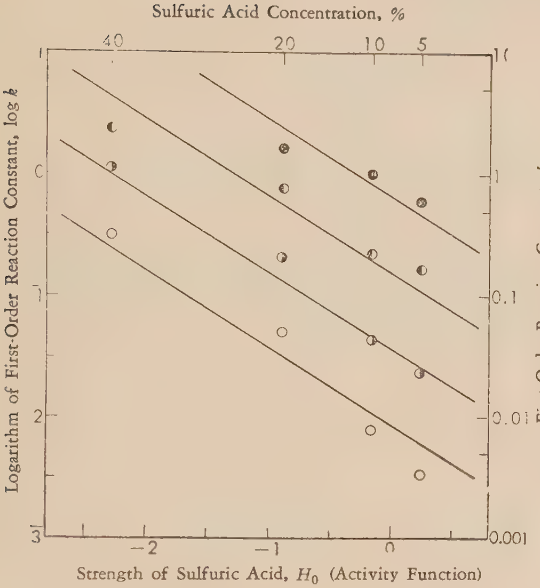


FIG. 5. Relation of First-Order Reaction Constant to Sulfuric Acid Strength in Hydrolysis of Soluble Glucose-Polymers at Different Temperature.

● 130°C ◐ 115°C ◑ 100°C ○ 87°C

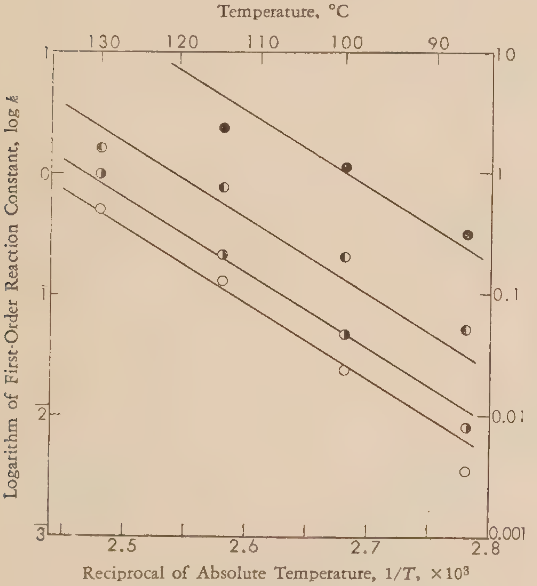


FIG. 6. Relation of First-Order Reaction Constant to Temperature in Hydrolysis of Soluble Glucose-Polymers with Sulfuric Acid of Different Strengths.

● 40% H_2SO_4 (H_0: −2.28) ◐ 20% H_2SO_4 (H_0: −0.89)
◑ 10% H_2SO_4 (H_0: −0.16) ○ 5% H_2SO_4 (H_0: +0.24)

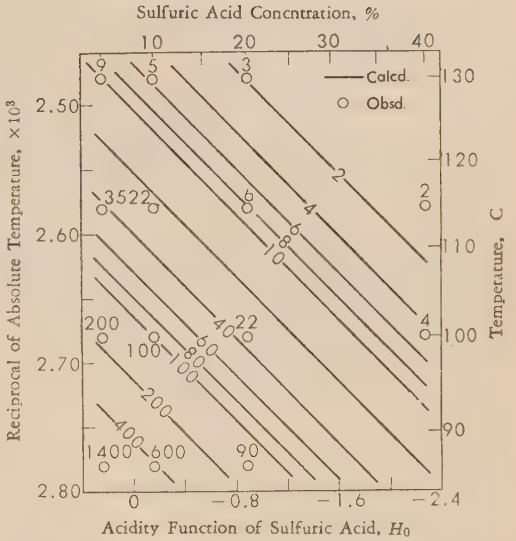


FIG. 7. Time of Complete Hydrolysis of Soluble Glucose-Polymers Expressed as Functions of Temperature and Sulfuric Acid Strength.

lines, -0.632, in Fig. 5 by 2.303. The energy of activation, $E=28400$ cal., is obtained by multiplying the slope of the straight lines, -6209, in Fig. 6 by -2.303×1.968. The value of H calculated from the data in Table II and the values of E and M are 1.61×10^{15}. Then, the hydrolysis rate of the soluble glucose-polymer in dilute sulfuric acid can be represented by the following equation:

$$k=1.61\times15^{15}\exp[-1.46\,C-\{28400/(RT)\}]. \quad (3)$$

While, on the other hand, the hydrolysis rate, k_c, of cellulose dissolved in concentrated sulfuric acid in concentrations from 51 to 57% at temperatures within the range of 15.5 to 45° C (1) is indicated accoding to the following equation:

$$k_c=2.60\times10^{15}\exp[-1.50\,C-\{29800/(RT)\}]. \quad (4)$$

Equation (3), corresponds approximately with equation (4); the values of H, M and E in equation (3) are close to those in equation (4) respectively. From these facts, it would seem that both concentrated and dilute sulfuric acid act upon cellulose and degraded cellulose in the same manner, provided they are dissolved.

The retention time required for the post-hydrolysis under different conditions are represented in Fig. 7 which is by the same procedure as the described in the previous report[1]. If the time required to split 99% of the initial glucose-polymers' bonds substitutes the retention time for completing post-hydrolysis, it is calculated by a method similar to the calculation of a half-life time. The time required for 99% decay is related to the reaction constant, k, by the equation:

$$2.303 \log 0.01 = -k t_{0.99}$$

therefore,

$$\begin{aligned} t_{0.99} &= 4.606/k \\ &= 1/(3.495 \times 10^{14} \exp[-0.632 \times 2.303\, C - \{28400/(RT)\}]). \end{aligned}$$

Fig. 7 represents the relation of $t_{0.99}$ to sulfuric acid strength and the temperature.

Acknowledgement. The authors wish to express their appreciation to members of Wood Saccharification Discussion Committee for their helpful suggestions extended during the course of this work.

Short Communications

The Chemistry of Ilexol. II.*

A Provisional Structure of Ilexol

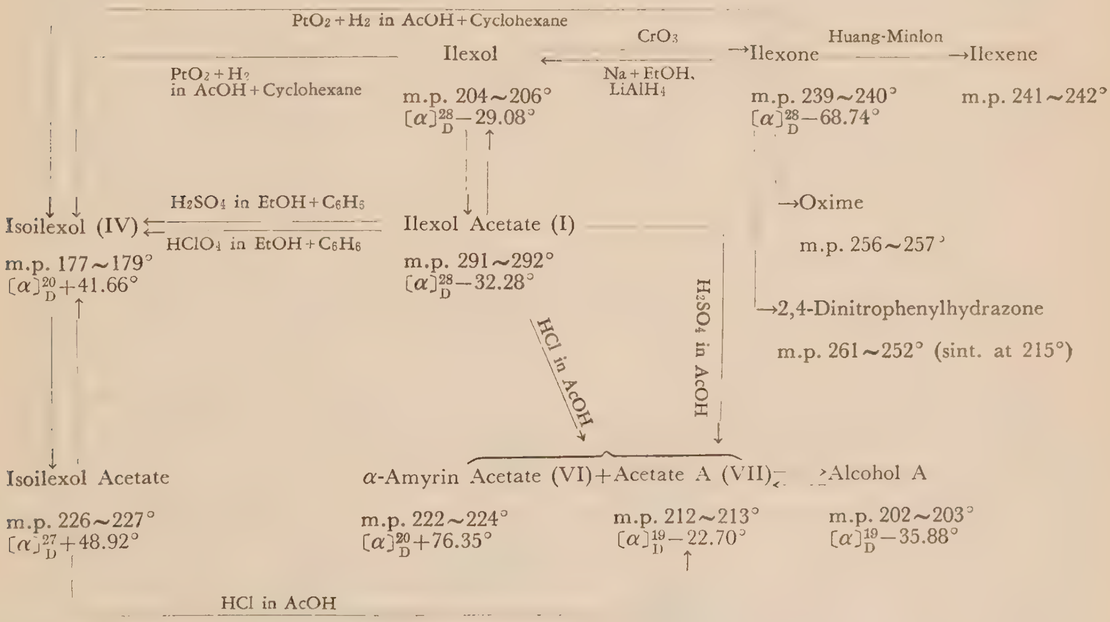
Sir:

It has now been shown that: (a) treatment with either perchloric acid or sulfuric acid of ilexol acetate in a mixture of benzene and ethanol, and (b) treatment with platinized hydrogen[1] of ilexol and ilexone[2] suspended in a mixture of glacial acetic acid and cyclohexane, affords an isomeric alcohol desginated as isoilexol, which has $C_{30}H_{50}O$, m.p. 177~178°, $[\alpha]_D^{20}+41.66°$ (c, 0.512, $CHCl_3$), giving acetate, $C_{32}H_{52}O_2$, m.p. 226~227°, $[\alpha]_D^{27}+48.92°$ (c, 0.511, $CHCl_3$).

Furthermore, it has been shown that: (a) treatment with hydrochloric acid and glacial acetic acid[3] of ilexol acetate affords α-amyrin acetate[4], $C_{32}H_{52}O_2$, m.p. 222~224°, $[\alpha]_D^{20}+76.35°$ (c, 0.572, $CHCl_3$), and in addition, an acetate (Acetate A), $C_{32}H_{52}O_2$, m.p. 212~213°, $[\alpha]_D^{19}-22.70°$ (c, 0.488, $CHCl_3$), which is hydrolyzed to an alcohol (Alcohol A), $C_{30}H_{50}O$, m.p. 202~203°, $[\alpha]_D^{19}-35.83$ (c, 0.385, $CHCl_3$); (b) treatment with sulfuric acid and glacial acetic acid of ilexol acetate, and (c) treatment with hydrochloric acid and glacial acetic acid[5] of isoilexol, affords only Acetate A, respectively.

Although all of the infrared spectra of

CHART I



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* Part I: This Bulletin, **21**, 274 (1957).

[1] cf. H. Wieland and W. Benend, *Ann.*, **544**, 1 (1943); H.E. Stavely and G.H. Bollenback, *J. Am. Chem. Soc.*, **65**, 1600 (1943); D.E.R. Barton and I.D. Cox, *J. Chem. Soc.* **1948**, 1354.

[2] K. Yagishita and S. Iseda, This Bulletin, **21**, 274 (1957).

[3] cf. J.M. Beaton, F.S. Spring, R. Stevenson and J.L. Stewart, *J. Chem.Soc.*, **1955**, 2131.

[4] K. Yagishita, This Bulletin, **21**, 160 (1957).

[5] cf. G. Brownlie, M.B.E. Fayez, F.S. Spring, R. Stevenson and W.S. Strachan, *J. Chem. Soc.*, **1956**, 1377.

ilexol, ilexol acetate, ilexone and ilexene[2] reveal a sharp absorption band at 819, 820, 817, and 817 cm^{-1} characteristic of the grouping $>C=C<^{H}$ respeccively, it has now been shown that the infrared spectra of both isoilexol and isoilexol acetate reveal no appreciable absorption band in this region respectively, indicative of the disappearance of the above grouping in each of their molecules.

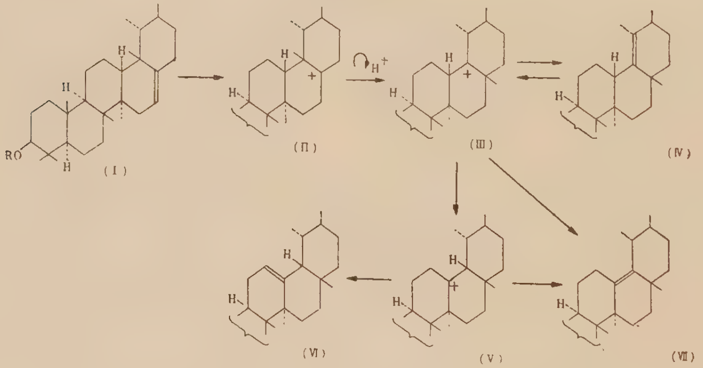
It is hereby noteworthy that ilexol and all its derivatives obtained so far, exhibit a positive color reaction with tetranitromethane respectively, indicative of the presence of at least one double linkage in each of their molecules.

On the basis of the foregoing results of experiments, it has now become evident that: (a) both ilexol and isoilexol are triterpenoid alcohols of the α-amyrin group, and (b) Alcohol A and Acetate A might well be identical with ursa-13(18)-en-3β-ol[6,7] and ursa-13(18)-en-3β-yl acetate[6,7] resepctively because of good agreement in their physical constants.

TABLE I

Compound		
Ursa-13(18)-en-3β-ol[6]	m.p. 201～202°	$[\alpha]_{D} -36^{\circ}$, -37°
Ursa-13(18)-en-3β-ol[7]	m.p. 204～205°	$[\alpha]_{D} -35^{\circ}$, -36°
Alcohol A	m.p. 202～203°	$[\alpha]_{D}^{19} -35.88^{\circ}$
Ursa-13(18)-en-3β-yl Acetate[6]	m.p. 213～215°	$[\alpha]_{D} -22^{\circ}$
Ursa-13(18)-en-3β-yl Acetate[7]	m.p. 214～216°	$[\alpha]_{D} -22^{\circ}$, -23°
Acetate A	m.p. 212～213°	$[\alpha]_{D}^{19} -22.70^{\circ}$

CHART II



Taking into account the foregoing facts, it is reasonable to assume that ilexol might well be formulated as Formula (I), which would be designated as ilexa-16-en-3β-ol hereafter.

Isomerization of ilexol into isoilexol (IV), Alcohol A [ursa-13(18)-en-3β-ol] (VII) and α-amyrin (VI) can only be explained by the aid of this reasonable formula.

Further elucidation of the chemical structure of ilexol is now underway, and will be reported in detail in this Bulletin as soon as possible.

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Received December 20, 1957

[6] J.M. Beaton, F.S. Spring, R. Stevenson and W.S. Strachan, *J. Chem. Soc.*, **1955**, 2610.

[7] J.D. Easton, W. Manson, and F.S. Spring, *ibid.*, **1953**, 943.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 2, p. 125～126, 1958]

A Crystalline Volatile Substance "Oryzarol" from Rice Plant, Possessing a Stimulating Effect on the Germination of Blast Mould Spore

Biochemical Studies on the Blast Disease of Rice Plant. Part XI.

Sir:

It is a very interesting problem: "why does the Blast mould especially attack rice plant". Previously, Kawamura and Ono1) made investigations of the germination of Blast mould spores in a petri dish which was set near by rice plants in a vessel and observed the germination and the appressorium formation of Blast mould spores to be remarkably stimulated by a certain volatile substance of rice plant. From this observation, they considered that this substance, existing in the dew drops on rice plant, should play a significant role when Blast mould spores make an attack this plant.

The present communication deals with the isolation and characterization of a crystalline volatile substance, possessing a remarkably stimulating effect on the germination of Blast mould spore, from rice plant.

Young rice plants (Norin No. 21) were cut, ground and steam-distillated.

From the ether extract of the steam distillate an effective oily substance was obtained, belonging to the neutral fraction. This substance was treated with alcohol and separated into easily soluble and insoluble fractions.

The alcohol soluble fraction, treated with ethanol and water, gives colorless needle crystals after cold storage for many days, being soluble in almost all organic solvents (especially easily soluble in ether, $CHCl_3$, CCl , ethylacetate, benzol and butanol) and almost insoluble in water.

The crystal, recrystallized from ethanol and water, melts at 44～46° C and possesses

TABLE I STIMULATING EFFECT OF ORYZAROL ON THE GERMINATION OF SPORES OF *Piricularia oryzae* CAVARA P_2.

Concentration of Oryzarol ppm	Total spores	Germinated spores	"χ^2" obtained	"χ^2" at 5% level of significance d.f.=1	Germination rate %
0 (Control)	359	99			27.6
100	316	213	106.6	⋙ 3.84	67.2
10	342	140	13.5	≫ 〃	40.9
1	432	175	13.4	≫ 〃	40.5
0.1	583	250	21.8	≫ 〃	42.9
0.01	447	172	8.3	> 〃	37.5

Test method: Drop culture (Incubated 5 hours at 27°C)

Age of the spores: 2 weeks (Cultured at 27°C on Misato-medium: soluble starch 1%, yeast extract powder 0.2%, agar 1.5%)

d.f.: Degree of freedom

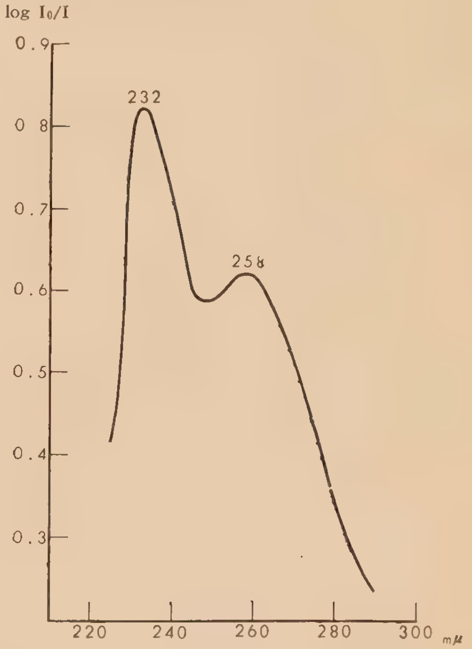

FIG. 1 Ultravioletabsorption spectra of Oryzarol (Ethanol solution)

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1) E. Kawamura and K. Ono, *Agriculture and Horticulture*; **21**, 101 (1946).

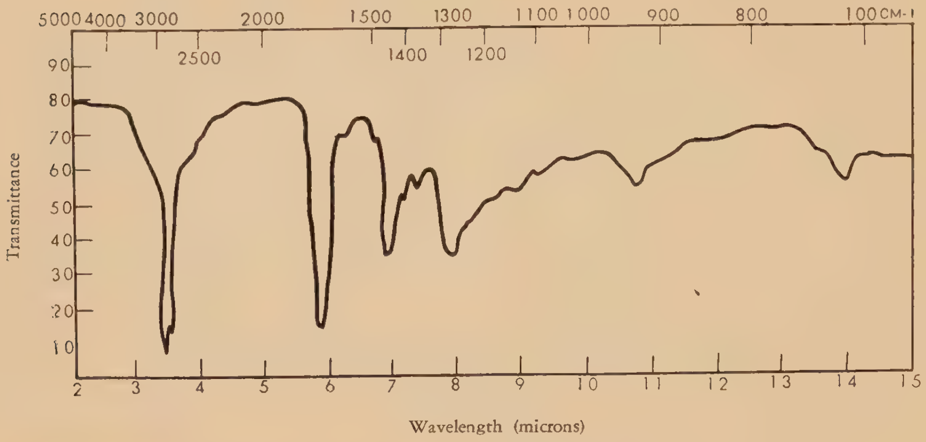

FIG. 2 Infrared spectra of Oryzarol (Phase: melt)

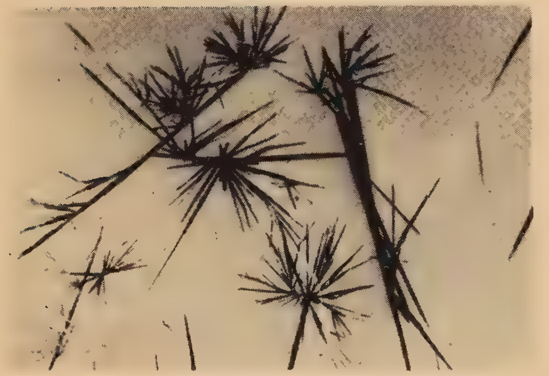

PHOTO I
Crystals of Oryzarol
(× 150)

a remarkably stimulating effect on the germination of Blast mould spore as shown in Table I. We named this substance "Oryzarol" and $C_{25}H_{44}O_3$ was assigned as its molecular formula from the analytical data and molecular weight determination: Found, C 76.52%, H 11.24%, M.W. 403 (Rast-Method); Calcd. for $C_{25}H_{44}O_3$, C 76.53%, H 11.22%, M.W. 392.

Oryzarol strongly smells like rice straws and gives a Liebermann's color reaction of sterol (red-vilolet). The ultraviolet and infrared absorption spectra of Oryzarol are shown in Figures 1 and 2.

The authors with to express their sincere thanks to Dr. T. Yabuta, Professor Emeritus of the University of Tokyo, Dr. K. Sakaguchi and Dr. Y. Sumiki, Professors of the University of Tokyo, for their encouragement and interest in this work and also to the Research Laboratory of Takeda Pharmaceutical Co. for the microanalyses and measurement of the infrared spectra.

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Received December 33, 1957

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 2, p. 127, 1958]

The Appositive Growth of Starch Granules in Beans as revealed by Autoradiography

Received Dec. 24, 1957

Sirs:

On the growth of reserve starch granules in tubers and grains, many speculations have been proposed, but as yet, it has been impossible to observe growth directly. Recently, Badenhuizen and Dutton[1] reported on the growth of C^{14}-labeled potato starch granules by gradual centrifugal deposition of starch layers (apposition). However, their interpretation of the autoradiograms obtained from radioactive whole granules of potato starch seems to involve several points to be discussed.

We were working on the same problem independently, and have obtained more clear-cut autoradiograms which gave better evidence towards the apposition theory of starch granules.

An amount of about 1,000 μC of C^{14} as carbon dioxide was assimilated by one of the leaves of an almost matured red bean plant (*Phaseolus angularis*) during the period of two hours, and starch granules were collected from its beans twenty-four hours later. About 10% of the original radioactivity was stored in the granules (0.485 g, 200 μC per gram).

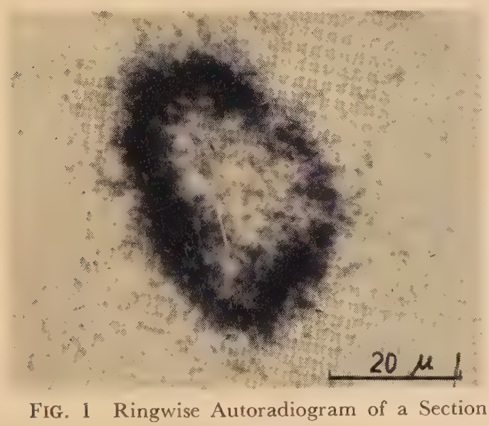

FIG. 1 Ringwise Autoradiogram of a Section of Red Bean Starch Granule.

The starch granules were then dried, embedded in methacrylate resin and sliced into sections of 0.5 micron in thickness using an ultra-thin microtome. A ribbon of the sections was transferred onto a slide glass, and the stripping emulsion method of autoradiography was applied to the sections. After sixty days' exposure, the film was developed. Many larger sections (exceeding approximately 20 microns, in diameter) gave ringwise autoradiograms as shown in Fig. 1, clearly indicating the appositive growth of the starch granules in bean. The section as observed in Fig. 1 was stained by iodine uniformly as shown in Fig. 2, indicating that there was no hole or fissure in the section.

Thus, it was confirmed that reserve starch granules in red bean grow by apposition of starch layers.

Details of this experiment will appear in the Memoirs of the Institute of Scientific and Industrial Research, Osaka University, 1958.

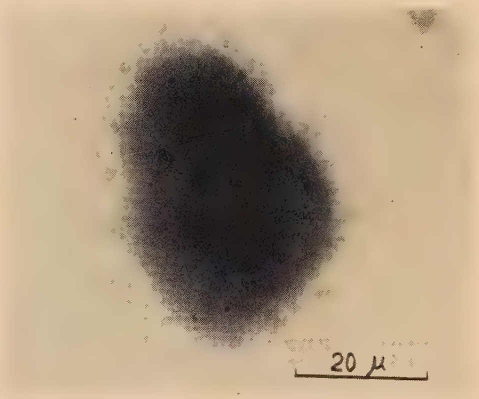

FIG. 2 The Same Section stained by Iodine.

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Osaka University

* Institute of Applied Microbiology, University of Tokyo.

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[1] N.P. Badenhuizen and R.W. Dutton: *Protoplasma*, 47, 156 (1956).

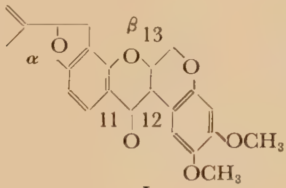
[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 2 p. 128~130, 1958]

Synthesen und Konfigurationsermittlung in Rotenoid-Reihe

IV. Mitteilung. Synthese des Rotenons

Sehr geehrter Herr Schriftleiter:

Die Strukturformel des Rotenons (I), welches besonders auf Fische und Insekten gifting wirkt, ist gleichzeitig von A. Butenandt[1], von F.B. LaForge[2] und von S. Takei[3] aufgestellt worden. Seither von verschiedenen Arbeitsgruppen sind bereits wichitige Vorarbeiten zur Synthese dieser Verbindung geleistet worden. In der vorliegenden Mitteilung möchten wir die erste Synthese des Rotenons berichten.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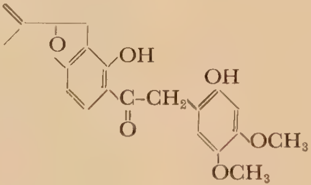
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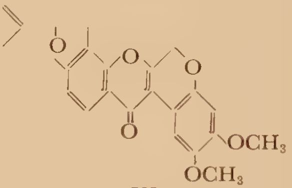
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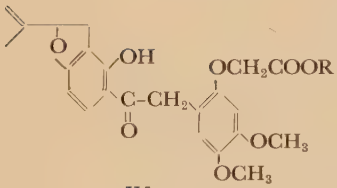
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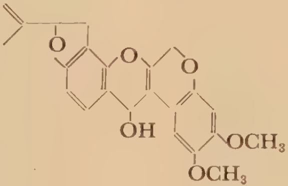
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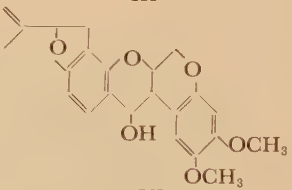
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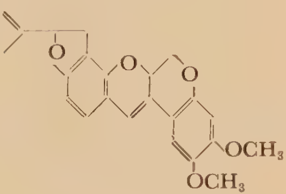
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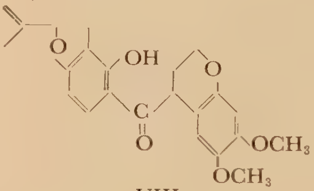
VIII

Durch Kondensation von Derritol (II) mit Bromessigsäure-äthylester unter Einwirkung von Natrium-äthylat erhielten LaForge und Mitarbb[4]. Dehydrorotenon (III) neben Derris-säure-äthylester (IV, $R=C_2H_5$). Danach stellten Takei und Mitarbb[3]. das Dehydrorotenon durch Erhitzen von Derris-säure (IV, $R=H$) mit Acetanhydrid und Natrium-acetat dar.

Da man das Dehydrorotenon zum Rotenon katalytisch nicht hydrieren kann, haben wir die Reduktion von Dehydrorotenon mit Zinkstaub in Eisessig versucht, aber es war Essig damit. Wir haben diesen Weg nicht weiter verfolgt, sondern als Alternative das Aufbauschema gewählt, welches aus Reduktion von Dehydrorotenon zur Verbindung (V) mit Komplex-Metall-Hydrid und anschliessende Umlagerung zum Rotenon besteht. Wider unseres Erwarten, geht das Dehydrorotenon bei der Behandlung mit überschussigem $NaBH_4$ in fast quantitativer Ausbeute in die amorphe Verbindung (VI) über. Analysenzahlen $C_{23}H_{24}O_6$ Ber.: C 69.7, H 6.1, Gef.:

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[1] A. Butenandt und W, McCartney, *Ann.*, **494**, 17 (1932).

[2] F.B. LaForge und H.L. Haller, *J. Am. Chem. Soc.*, **54**, 810 (1932).

[3] S. Takei, S. Miyajima und M. Ohno, *Ber.*, **65** 1041 (1932)

[4] F.B. LaForge, H.L. Haller und L.E. Smith, *J. Am. Chem. Soc.*, **53**, 4406 (1931).

C 68.8, H 5.7. Es handelt sich um den zugehörigen Alkohol des Rotenons, daher möchten wir diese Verbindung als Rotenol[5] bezeichnen. Behandelt man den Rotenol mit Salzsäure in wässrigem Alkohol geht es unter Abspaltung von 1 Mol Wasser in fast quantitativer Ausbeute in die Verbindung (VII) vom Schmp. 160–161° über. Analysenzahlen $C_{23}H_{22}O_5$ Ber.: C 73.0, H, 5.9, Gef.: C 73.0, H 5.8. Wir nennen diese Verbindung Desoxy-Δ^{11}-dehydro-rotenon. Die Struktur von (VI) und (VII) wird wie folgt festgestellt. Durch Reduktion mit $NaBH_4$ liefert das Rotenon in guter Ausbeute eine amorphe Verbindung, dessen IR-Spektrum in Chloroform mit dem der aus Dehydrorotenon erhaltenen Verbindung (VI) ganz gleich ist. Analoger Weise versetzt, liefert dieses amorphe Produkt das Desoxy-dehydro-rotenon. Analysenzahlen Gef.: C 72.9, H 6.0, Mischschmp. 160–161°. Insbesondere stimmt das IR-Spektrum in Nujol Paste ganz überein. Da die Konfiguration des C_{13}-Atoms bei der Reaktionsbedingung von Rotenol→Desoxydehydro-rotenon nicht umkehrt, haben beide Rotenole (der eine aus Retenon, der andere aus Dehydrorotenon) nicht nur dieselbe Struktur, sondern auch die identische Konfiguration. Es ist auch zu schliessen, dass der Zusammenhang zwischen C_{12} und C_{13} die stabilere d.h. *trans*-(threo-) Konfiguration sei, mit Vorbehalt, dass die Konfiguration des C_{12}-Atoms beim Vorgang von Rotenon→Rotenol nicht umkehre. Durch Oxydation vom Rotenol mit Aceton unter Einwirkung von Aluminium-isopropylat in Benzol erhalten wir fast farbloses Öl in guter Ausbeute, welches das wesentlich identische IR-Spektrum mit Rotenon in Chloroform bezeigt. Gibt man ihm den Methanol zu, scheiden die farblose Blättchen vom Schmp. 128–9° (nach Umkristallisieren aus Methanol) aus. Analysenzahlen $C_{23}H_{22}O_6$ Ber.: C 70.0, H. 5.6, Gef.: C 69.9, H 5.7 IR-Spektrum dieser Blättchen in Nujol Paste stimmt mit dem des Rotenons nicht überein, sondern mit demjenigen des sogenanten Mutarotenons[6] ganz überein. Cahn und Mitarbb[7]. berichteten, dass durch Behandlung von Mutarotenon mit Kohlentetrachlorid das natürliche l-Rotenon isoliert werden konnte. Wir erhalten das l-Rotenon in fast quantitiver Ausbeute durch 5-minutiges Erhitzen auf 190° und anschlessende Umlösen aus heissem 95-proc. Alkohol. Das bedeutet die doppelte Ausbeute gegenüber derjenigen von Cahn und Mitarbb. Das von uns synthetisch dargestellte Rotenon schmilzt auf 162–3° (Misch-schmp. 162–3°), und sein IR-Spektrum in Nujol Paste stimmt mit dem des natürlichen Rotenons ganz überein. $[\alpha]_D$ −226 (Benzol). Analysenzahlen $C_{23}H_{22}O_6$ Ber.: C 70.0, H 5.6, Gef.: C 69.9, H 5.9. Dieser Befund deutet wieder hin, dass das Rotenon die stabile Konfiguration von C_{12} und C_{13} habe. Bei der Reduktion von Dehydrorotenon mit $LiAlH_4$ erhalten wir eine neue amorphe Verbindung (V) (Analysenzahlen $C_{23}H_{22}O_6$ Ber.: C 70.0, H 5.6, Gef.: C 69.0, H 5.9), die wir als Dehydrorotenol bezeichnen. Wir können diese Verbindung von Rotenol dadurch verscheiden, dass IR-Spektrum der ersten OH-Absorption in 2.91 μ und charakteristische komplizierte Absorptionen in C=C Bereich hat während die andere 2.79 μ (OH) und starke Absorption in 8.78 μ. Beide Alkohole verhalten sich sehr verschieden bei der Chromatographie auf Alumina. Die erwartete Umlagerung vom Dehydrorotenol zum Rotenon läuft weder durch Behandlung mit Salzsäure noch durch alkoholische Natronlauge ab. Andererseits erhalten wir bei der Chromatographie auf aktiviertem Alumina (Benzol 9: Aceton 1) farbloses Öl in sehr geringer Ausbeute, welches das wesentlich identische IR-Spektrum mit Rotenon in Chloroform bezeigte. Es ist von Interesse festzustellen, ob die Umlagerung durch Alumina bewirkt wird, ob ein Teil des Dehydrorotenons von Anfang an die 1,4-Anlagerung von $LiAlH_4$ erleidet. Es gelingt uns noch nicht das kristallinische Rotenon aus diesem Öl zu isolieren.

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[5] Ob gleich der Name „Rotenol" schon von Butenandt zu einer Verbindung (VIII) gegeben worden ist, möchten wir nun die letztere als C_{13},O_β-Dihydrorotenonol bezeichnen.

[6] Durch Einwirkung von alkoholischer Kalilauge, Kaliumcarbonat, Natriumacetat usw. haben Cahn und Mitarbb. eine molekulare Verbindung „Mutarotenon" erhalten, welches aus gleichen Mengen von Rotenon und sein Stereoisomer besteht. unstabiler Schmp. 146°, $[\alpha]_D$ −83 (Benzol).

[7] R.S. Cahn, R.F. Phipers und J.J. Boam, *J. Chem. Soc.*, **1938**, 513.

Diese Mitteilung bedeutet die Synthese des Rotenons (I) aus Derritol (II).

Herren Prof. Dr. R. Yamamoto, Prof. Dr. Y. Sumiki und Dr. S. Tamura danken wir an dieser Stelle für die Unterstützung und Förderung dieser Arbeit. Wir danken Hrn. K. Aizawa für die freundliche Aufnahme und Diskussion des IR-Spektrums, Fr. K. Satô. Frl. M. Suzuki und Frl. I. Isobe für Mikroanalysen. Wir sind Hrn. T. Yamamoto, Kakenkôgyô G.m.b.H., für die bereitwillige Überlassung von Rotenon zu bestem Dank verpflichtet.

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Eingegangen am 2, Januar 1958

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Wood Extractives. Part VII. On the Wood Constituents of *Morus bambycis* Koidz. (1) (*P. 1~4*)

By Tamio KONDO, Hiroyuki ITO, and Motoshige SUDA

(*Government Forest Experiment Station, Meguro, Tokyo*)

Two polyhydirc phenols, i.e., morin (3,5,7,2′,4′-pentahydroxyflavone) and oxyresveratrol (*trans*-2,4,3′,5′-tetrahydroxystilbene) have been isolated and identified. The latter compound was identical with cudranin, previously isolated from the woods of *Cudrania triloba* and *Morus alba* by Tazaki and Suzushino et al. Furthermore, by paper chromatographic procedures the following sugars have been detected: glucose, mannose, xylose, rhamnose, and two unidentified oligosaccharides.

On the Reduction Products of Rotundifolone with Lithium Aluminum Hydride (Studies on the Essential Oil of *Mentha rotundifolia*, Part III). (*P. 5~9*)

By Sumio SHIMIZU

(*Laboratoay of Agricultural Chemistry, Faculty of Agriculture, Shinshu University*)

The structure of rotundifolone was established to be 1-methyl-4-isopropylidene-1,2-epoxy-cyclohexanone-3 previously. By the reduction with lithium aluminum hydride, this ketone yielded two diols, which were shown to be completely identical with the diols from "mintglyoxal", the principal component of Indian spearmint oil. These diols were demonstrated to be 1,3-diol, since they were ~~gave~~ negative to periodic acid test and produced piperitone on chromic acid oxidation. These facts support the epoxide ketone structure proposed by the present author, but do not the cyclopropane glyoxal presented by Chakravarti et al., in *Perfumary Essent. Oil Record*, 256 (1955); 62 (1957).

Studies on Activities of Baker's Yeast. Part II. Relationship between the metabolic Activity and the Dough Rising Power. (*P. 9~11*)

By Tomotaro SATO, Yasuo TANAKA, and Hiroko MATSUSHITA

(*Food Research Institute, Administry of Agriculture and Forestry.*)

To obtain fundamental information concerning the activities of Japanese baker's yeasts, their metabolic activities and the relationship between the activity and dough rising power were investigated. The mean values of Q_{O_2},$Q^{O_2}_{CO_2}$ and $Q^{N_2}_{CO_2}$ were 103, 47, and 277 respectively. These values compared with those of foreign baker's yeasts, showed a higher Q_{O_2}-value and a much lower, $Q^{O_2}_{CO_2}$ whereas no marked difference was found in the activity of anerobic fermentation. Both Q_{O_2} and $Q^{N_2}_{O_2}$ were found to be correlated significantly with ordinary dough rising power; however, no correlation was found between sweet dough (containing much sugar) rising power and the metabolic activity.

Therefore sweet dough rising power seemed to be controlled by a mechanism different from that of ordinary dough rising power.

Studies on the Activities of the Baker's Yeast. Part III. Changes of the Metabolic Activity and the Dough Rising Power during the Manufacture of Baker's Yeast. (*P. 11~14*)

By Tomotaro SATO, Yasuo TANAKA and Yoshiko KOYANAGI

(*Food Research Institute, Administry of Agriculture and Forestry.*)

During the manufacture of baker's yeast, Q_{O_2} assumed a higher value with the increase of the aeration rate, but it turned to a constant level when the wort was aerated more violently. $Q^{O_2}_{CO_2}$ on the other hand, showed a contrary tendency.

$Q^{N_2}_{CO_2}$showed a very high value in the early-stage of anerobic seed culture, but decreased gradually when the aeration rate was increased. The change of $Q^{N_2}_{CO_2}$ was however not so remarkable as Q_{O_2} or $Q^{O_2}_{CO_2}$.

Meyerhof's quotiety (M.Q.) was found to be almost constant during the whole process.

Dough rising power (total activity) also remained almost constant during the whole process, but in the anerobic stage the type of fermentation was that of a slow-fast type and which turned to the fast-slow type in the aerobic stage.

The change of sweet dough (containing much sugar) rising power was very remarkable, namely, baker's yeast in anerobic culture could not ferment sweet dough, but in the aerobic stage it could ferment sweet dough very well.

Studies on the Chemical Composition and Quality of Silage. Part II. On the Nutrient Content and Quality. (*P. 14~19*)

By Hiroshi SUTOH

(*Laboratory of Animal Feeding, Fuculty of Agriculture, Okayama University, Japan*)

Samples of silage obtained from different districts in Japan, were statistically analyzed for the purpose of finding the relationship between the nutrient content and quality, and also for discovering the fundamental principle of silage-making.

The contents of moisture, crude protein, ether extract, crude fiber, and crude ash, as well as pH value, were determined in the usual manner for each sample.

A significant negative correlation, $r=-0.346$, was found to exist between the dry matter content of silage and the subsidence of silage mass in the silo during the storage ($p<0.01$). A positive correlation, $r=+0.451$, was found between the pH value and the moisture content of silage ($p<0.001$).

Analyzing the data obtained from silages of good quality and those of poor quality, the author found that the contents of dry matter and nitrogen-free extractives in the former were significantly higher than those in the latter, while the relation of contents of crude fiber and crude sah was contrary.

Studies on the Aspergilli for Saké Brewing. Part IV. On the Brewing of Saké. (*P. 19~22*)

By Hideya MURAKAMI

(*Brewing Experiment Station, Tax Administration Agency*)

A great variety of Japanese saké can be made not only by the different conditions of brewing, but also by the different kinds of molds *Aspergillus flavus-oryzae* groups. The author studied the relations between the properties of the molds and the qualities of saké. In this report he described the kinds of molds, method of brewing and the chemical components of saké. Especially he found that the oxido-redux potential of saké correlated closely the oxygen requirements or the oxidase activity of the molds.

Studies on Continuous Fermentation. Part VI. Continuous Fermentation of Molasses with a Combined System of Agitated Fermentor and Flow Pipe. (*P. 26~29*)

By Kiyomoto UEDA

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The fermentation cycle in continuous multifermentor system was investigated, and it was obsered that the cycle time is prolonged especially, in the latter fermentors where the mashes were almost fermented out.

A new continuous system was devised to reduce the cycle time furthermore, and preliminary experiments were carried out previous to the pilot plant operation, using a small equipment which was connected with a 10-liter glass jar fermentor and a 4-liter glass flow pipe.

In the continuous fermentation by this new system, about 40% of cycle time could be reduced in comparison with batch fermentation.

Butanol Fermentation. Part XVI. Behavior of Redox System. (*P. 29~33*)

By Motoyoshi HONGO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

Using a Weizmann type butanol producing strain, *Clostridium acetobutylicum* 314, experiments to investigate the behavior of each metabolic redox system in this fermentation by the use of oxidation reduction potential of the culture were performed as follows:

1. By confirming that methylen blue added to the washed living cell suspension was reduced to fade, by passage of hydrogen gas through it, the presence of the $rH_0=0$ system ($H_2 \rightleftarrows 2H \rightleftarrows 2H^+ + 2e$ system) in this bacteria was proved.
2. The courses of changes of rH, pH, acidity and gas evolution during the fermentation of sucrose were studied.

On the basis of these curves, especially the rH curve, and rH_0 of metabolic redox systems taking part in this fermentation (viz. triose phosphate oxidizing system $rH_0=0.8$, acetic acid and acetone forming system accompanied with $rH_0=0$, C_4-compounds reducing system (butyric acid and butanol forming system) $rH_0=4.8$, acetaldehyde-ethanol system $rH_0=7.6$, and $rH_0=0$) the behavior of each redox system was investigated.

From this investigation it is comprehended that this fermentation process does not follow such a simple pathway as alcohol fermentation or homo lactic acid fermentation, but each redox system, being concerned in this metabolic reaction in itself, acts as a regulator to normally maintain the dynamic equilibrium of this metabolism which is apt to deviate from the physiological rH level of this bacteria.

Studies on the Microbiological Assay of Vitamins and Amino Acids by the Pulp Disc Method. Part I. On the Assay of Several B-vitamins by the Pulp Disc Method. (1) (*P. 33~37*)

By Hisao KOJIMA, Yutaka MATSUYA, Hiroko OZAWA, Makoto KONNO, and Teijiro UEMURA.

(*Department of Agricultural Chemistry, Faculty of Agriculture, To~~kyo~~hoku University*)

Microbioassay of B-vitamins and amino acids with the micro-organisms is ususlly carried out using test tube. These methods are rather complicated and unprofitable for routine work, because many troublesome operation are required in the procedure. Then, attempting to make the assay procedure much easier and more simplified, it was determined whether or not the pulp disc method was applicable to the microbioassay. When an ordinary pulp of 9 mm diameter was used, good diffusion of the materials tested into the assay medium-agar layer occurred, which caused a defined growth zone of the micro-organisms under favorable conditions.

The pulp disc method for assay of several B-vitamins with lactobacilli or yeast was precisely investigated, and favorable conditions for producing sharply defined zones have been described. The length of the diameter of the growth zone corresponded very well to the concentration of the substances tested, and a linear co-relationship between them was obviously discerned. The assay range of the concentration of B-vitamins by this method was wider than that of the tube technique, and this method proved ratter more profitable to routine work than the latter techniques, although the former method was nore insensitive to a smaller concentration of the test materials. The growth zone obtained was much affected by the procedure conditions such as inoculum density, agar concentration, depth of agar medium, and culture period, and assay conditions suitable for obtaining the results with a good reproducibility are described.

Studies on the Microbiological Assay of Vitamins and Amino Acids by the Pulp Disc Method. Part II. On the Assay of Several B-vitamins by the Pulp Disc Method. (2) (*P. 38~42*)

By Hisao KOJIMA and Yutaka MATSUYA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

Bioassay of biotin, pantothenate, niacin, pyridoxine, and thiamine respectively, contained in several kinds of natural material by this method was carried out under the procedure conditions previously determined. The assay results obtained were compared with those by the tube technique. The cultured broth of Rhizopus sp., water-extracts of Rhizopus mycelia, and of Rhizopus-Koji, yeast extracts, liver extracts, and rice bran digest, respectively, were analyzed. The relation between the concentration of samples tested and diameter of the growth zone obtained was quite linear in the same manner with that obtained, in the case of testing known vitamins. Reproducibility of assay results were well established. The known amounts of vitamin added to the sample were recovered in about 90–110%. Comparing the results obtained by this method with those by the tube assay procedure carried out in parallel, the differences in assay values according to each technique were observed but the maximum range of them was about ±20%. Assayed values in the standard series varied within the range of ±6%, except in the assay test of pantothenate. The influence of NaCl on the assay results by the pulp disc method were also investigated and even a concentrated salt solution such as in 20% did not cause any disturbance in this assay procedure. Advantage to this pulp disc method is discussed as compared with the tube technique and some caution required in carring out this method is described.

Studies on the Abnormality of Plaque Formation by Bacteriophage of *Bacillus subtilis*. Part I. Its Occurrence and Some Findings. (*P. 42~44*)

By Shukuo KINOSHITA, and Takuji OKUMURA

(*Kyowa Fermentation Industy Co. Ltd. Tokyo Research Laboratory*)

An abnormality of plaque formation was observed in the *B. subtilis* PCI 219 (a susceptible host strain) and temperate phage L_1-Th system. Normal growth was obtained and no plaques were found when the host was cultured with high concentration (titer, $>10^6$/ml) of the phage. On the contrary, plaques appeared when diluted phage (titer, $<10^3$/ml) was used.

This is an abnormal phenomenon in the light of the action of the phage. The phenomenon is especially marked when the culturing temperature is high 37°C) or incubation time is long (80 hrs.).

Studies on the Abnormality of Plaque Formation by Bacteriophage of *Bacillus subtilis*. Part II. Investigations on the Cause of the Abnormality. (*P. 44~48*)

By Shukuo KINOSHITA and Takuji OKUMURA

(*Kyowa Fermentation Industy Co. Ltd. Tokyo Research Laboratory*)

To elucidate the cause of the abnormal phenomenon, several factors were studied. It was observed that the phage (L_1-Th) infected cells would not form plaques even in contact with the diluted phage (titer, $<10^3$/ml). Therefore lysogenization of the host cells was supposed to be the most probable factor responsible for abnormality. This was verified by the fact that cells (or spores) obtained from plates which showed the abnormality were invariably lysogenized.

The mechanisms of the abnormality were considered.

On the Production of Hydrogen Sulfide by Saké-Yeast. Part I. Pantothenic Acid Deficiency and the Production of Hydrogen Sulfide. (1) (*P. 49~54*)

By Ryoji KODAIRA, Yutaro ITO, Teijiro UEMURA

(*Faculty of Agriculture, University of Tohoku*)

In Japan, hydrogen sulfide accumulation during the Saké-brewing process has been often observed. We have occasionally observed hydrogen sulfide production in pantothenate deficient culture of Saké Yeast, Kyokai No. 6, which essentially requires pantothenate for its rapid growth. Then, the hydrogen sulfide production by pantothenate deficient Saké Yeast has been investigated in detail.

The Saké-yeast was cultured in the modified hyduck medium, to which $(NH_4)_2SO_4$ was added together with asparagine as the nitrogen source and also, a necessary amount of pantothenate was added. When pantothenate was added sufficiently, 200 μg/l, to the medium. a discernible amount of hydrogen sulfide was undetected throughout the cell growth. In contrast, pantothenate being added deficiently (10–15 $\mu g/l$) to the medium, ca. 10 μg of Hydrogen sulfide per ml of medium once accumulated at the early-stage of logarithmic growth phase, and then disappeared, if not at all, when the cell growth attained to maximum.

When the yeast was cultured in pantothenate deficient medium, which, however, contained casamino acid in the place of asparagine of the above Hyduck medium, hydrogen sulfide production did not occur any more. As hydrogen sulfide production by the pantothenate deficient yeast might assumably be originated from the sulfate present in the medium and inhibited by the concomitant presence of certain kinds of amino acid therein, the reversal effect of an individual amino acid on H_2S production from the sulfate in the pantothenate deficient culture was investigated. From the results so far obtained, H_2S production in the pantothenate deficient culture was almost entirely inhibited by the respective addition of methionine and leucine to the medium.

In contrast, glutamic acid, asparatic acid and alanine, respectively, which are directly derived from the members of TCA cycle, showed rather more favorable effect on H_2S production than the other amino acids,

Studies on the Bulk Sweating of Japanese Native Cigar Tobacco "Nambu". Part III. Changes of the Tensile Strength and the Elongation of Tobacco Leaves Taking Place in the Bulk Sweating.

(*P. 54~58*)

By Isao KANAI
(*Central Research Institute, Japan Monopoly Corporation*)

In a preliminary investigation[1] showed that the tensile strength of "Nambu" tobacco leaves, measured by the Schopper tensile strength tester for cigarette paper, decreases during bulk sweating. The tester employed was, however, unsuitable for measuring a sample of low tensile strength. Then, in the present paper, the elongation as well as the tensile strength of "Nambu" tobacco leaves has been investigated by an autorecording tensile strength- and elongation tester, newly designed based on the priniple of an air-micrometer.

The tensile strength both in the lateral margin and base parts was smaller than that in other parts.

The higher moisture content of the leaves promotes on their elongation as well as the tensile strength. But no definite relation was proved between the two elements.

Both the tensile strength and the elongation of the leaves decreased during sweating. The decreasing rate in the forced sweating was larger than that in the natural one. Furthermore, the fermented leaves of various grades appeared to become relatively uniform in these two elements.

Studies on the Organic Acids in Japanese Grapes and Wines. Part III. On the Balance Sheet of Organic Acids in the Wines Fermented with the Grape Skins.

(*P. 58 ~62*)

By Tadae KUSHIDA and Chiaki MARUYAMA
(*Research Institute of Fermentation, Yamanashi University*)

The organic acids contained in the various table wines were determined by means of the silica gel partition chromatography.

The wines made from crashed grapes were always higher in pH and lower in malic and tartaric acid contents than the wines from free run juice. The former also usually contained more acetic, succinic and lactic acids than the latter in the commercial production, but the difference did not appear in the experimental one.

During fermentation the decrease of malic acid probably induced by the enzymic actions of the grape skins was almost entirely inhibited, when the grapes were preheated at 100 C for 3 seconds. Although the decrease of tartaric acid and the increase of pH, probably caused by the precipitation of bitartrates and the buffer action of the skins, were slightly promoted by the heating.

It was also found that malic acid was commonly destructed and a notable amount of lactic acid was formed during one year storage of wine from crashed grapes, whereas such malo-lactic fermentation did not occur in the one obtained from free run juice in the same period.

Effect of Nutritional Conditions on the Multiplicaton of a Streptococcus Phage

(*P. 68~73*)

By Takichi KANENO, Sadao IWANO, and Kakuo KITAHARA
(*Institute of Applied Microbiology, University of Tokyo*)

Effects of nutritional substances in suspending media on the multiplication of bacteriophage LP-4 acting on *Streptococcus faecalis* R were studied. The phage proliferated fairly well in a synthetic medium conventionally used for the host organism, when a small amount of calcium chloride was supplemented. Single step growth experiments demonstrated that active phage synthesis was carried out in a simple mixture of glucose and eighteen amino acids, indicating much less importance of the other materials. The omission of sugars available for the bacterial growth or of amino acids from the complete medium resulted in no phage synthesis, although, in some experimental conditions, even these effects were not significant. Vitamins and purine-pyrimidines are also shown to be also essential in the absence of added amino acids.

Amino Acid Metabolism of the Brewing Yeast. Part II. Ammenia Secretion in the Presence of Inhibitors.

(*P. 73~78*)

Shiro CHIHARA and Teijiro UEMERA

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[1] I. Kanai and J. Kobata, *J. Agr. Chem. Soc. Japan*, **31** 304 (1957).

(*Faculty of Agriculture, Tohoku University*)

To reveal the ammonia acceptor system functioning in the anaerobic metabolism of tyrosine by saké yeast, the authors determined the variation of the intracellular amino acid pool during 3 hours' incubation of the yeast in tyrosine medium and found that the cell pool was then markedly deprived of arginine and glutamic acid.

Assuming that the glutamic acid system may be the first ammonia acceptor system, the effects of various analogues of glutamine-glutamic acid system and of ammonia system on the cell growth, Ehrlich's reaction, and ammonia secretion were investigated, and γ-glutamylhydrazide was recognized to be the effective inhibitor of the ammonia-acceptor system when tyrosine was metabolized by saké yeast.

On the Halophilic Character of Shoyu-Yeast. Part III. Nutritional Factors Affecting the Halophilic Behaviour of the Yeast. (2) (*P. 79～82*)

Masahiro SATO and Teijiro UEMURA
(*Faculty of Agriculture, Tohoku University*)

A halophilic Shoyu-yeast, *Zygosaccharomyces major* st. Kikkoman, was found to require several kinds of amino acids, especially methionine and cystine other than inositol and Ca-pantothenate for its normal growth in the absence of 10% NaCl. In contrast, however, in the ~~absence~~ of NaCl any kind of amino acid were not required. presence

Under the cultural conditions employed it was indicated that S-containing amino acids such as methionine and cystine had a far promoting effect on halophilism of the yeast and in the case of the culture deficient in such amino acids growth promoting effects of inositol and choline became much enhanced.

From the results obtained, it was assumably mentioned that a metabolic system in which metabolites such as inositol, choline and S-containing amino acids play an essential role, appeared to be more important for the mechanisms of yeast halophilism.

On the other hand, S-containing amino acids proved to be partialy replacable by either of PABA, folic acid, niacin, pyridoxine and choline. However, each of them except PABA showed an inhibitory effect on growth in the presence of methionine and cystine in contrast with PABA which in turn, has no such effect under the same cultural conditions.

Synopses of the Articles ~~appeared~~ printed in NIPPON NÔGEI-KAGAKU KAISHI
(in Japanese)
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Digestive Adsorption of Protein in Swine. Part I. (*P. 83～86*)

By Shikao ISHIKAWA
(*Faculty of Agriculture, Mie University*)

The relative absorption of protein from different segments of the intensitive was determined on a 2 per cent crude protein ratio, which was spectrophotometrically analysed using chromic oxide as the indicator.

Table The Rate of Absorption of Protein from the Intestive

	small intestive	cecum	colon	rectum
1st Exp.	%	%	%	%
Crude protein	64.0	68.4	79.0	79.3
True protein	78.0	78.4	81.7	82.1
Organic matter	52.8	56.8	68.1	70.3
2nd Exp.				
Crude protein	63.6	68.8	76.4	77.6
True protein	71.4	73.5	80.0	79.5
Organic matter	51.6	54.1	70.8	64.3

Results indicate means for two pigs of a litter 8 months old in the 1st exp., and 12 months old in the 2nd.

The data indicate that almost 80 per cent of the digestible protein is absorbed from the small intestive, and 18 per cent from the initial-half portion of the large intestive.

Studies on the Amylase Formation by Bacteria. Part III. Factors in Natto-Extract Effective in Accelerating Amylase Formation. (*P. 86～90*)

By Shigeo FUKUDA
(*Botanical Institute, Faculty of Science, University of Tokyo and Biological Institute, Nihon Medical College*)

In the course of this series of studies on the amylase formation in *Bacillus subtilis* var. *amyloliquefacus*, it was noticed by the writer that the addition of a small amount of Natto-extract to the culture medium markedly accelerated the production of the bacterial amylase in the medium. An increase in amylase level was also observed on addition of the extract to bacterial cells suspended in a synthetic medium containing phosphate and maltose, in which the organism remained incapable of growth most plausibly because of the high concentration of the buffer used (Fukumoto and Yamamoto, 1952). For the purpose of obtaining the effective principle in a more purified state, the Natto-extract was fractionated by means of paper partition chromatography, as well as the ordinary method for amino acid analysis using butyric acid *n*-butanol and *n*-propanol as extracting solvents (Darkin, 1920, Przylecki and Kasprzylek, 1937). It was discovered that the effective principle was contained in a fraction comprising basic amino acids and peptides. Several basic amino acids such as arginine, histidine and lysine were also found to be active in accelerating the amylase formation under investigation, but none of them could compete a superior effect of the Natto-extract when compared on the dry weight basis. The same was true with the mixtures of amino acids thus for studied. The efficiency of Natto-extract could not be exclusively accounted for by its content of amino acids, since the hydrolysate of the original extract (heated for 3 hours in 3.0% HCl at 151°C) was shown to be active as compared with the original one in accelerating the amylase formation.

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Studies on the Aspergilli for Saké Brewing. Part V. On the Relations Between the Properties of the Aspergilli and the Qualities of Saké. (*P. 91~95*)

By Hideya MURAKAMI

(*Brewing Experiment Station, Tax Administration Agency*)

After the morphological and the physiological researches of molds and the experiments of brewing, he distinguished the molds into three types by their oxygen requirements, that is the aerobic, the anaerobic and the intermediate types; similarly the saké into three types by their oxido-redux potential, that is the oxidative, the reductive and the intermediate types. The interdependence between the molds and the qualities of saké was summarised as the next table.

Types of Saké	Types of Molds	Qualities of Saké			
		Oxido-redox potential	Color	Acids, Amino acids	Smell
Oxidative	Aerobic	High	Deep	Much	Common
Reductive	Anaerobic	Low	Pale	Little	Abnormal, sometimes fruit-like.
Intermediate	Intermediate	Variable	Variable	Variable	Variable

The browning of kōji and saké lees took place more quickly when the aerobic types of Aspergilli were used than the anaerobic or the intermediate types were used. By these results, the author regarded the aerobic type of molds as the typical Aspergilli for saké brewing. These types of molds were also distinguished by the production of chestnut-like aroma in the cultural medium and named by him conveniently *Aspergillus oryzae* var. *saké* after Dr. K. Sakaguchi's nomenclature.

Studies on the Aspergilli for Saké Brewing. Part VI. Experimental Considerations on the Various Phenomena of Saké-brewing from the View Point of the Properties of *Aspergillus oryzae* var. *saké*. (*P. 96~100*)

By Hideya MURAKAMI and Mitori KAWAI

(*Brewing Experiment Station, Tax Administration Agency*)

When the aerobic types of molds (i.e. *Aspergillus oryzae* var. *saké*) are used in the saké brewing, all brewing processes usually progress favourably, for instances, the kōji making is very easy, the fermentation is vigorous, the denaturation of saké in storage is little, but when the anaerobic types are used it is contrary. In tnis repot, the authors described the relations between the forms of conidiophores and the respiration of molds, comparing the oxygen requirements of *Aspergillus oryzae* var. *saké* with other various kinds of fungi, and gave an adequate explanations for the various phenomena occurring in saké brewing:

(1) The molds with abnormal types of conidiophores require the less amount of sugars for the maintenance of life mechanism than the ones with normal types.

(2) The amount of oxygen requirements of the *Aspergillus oryzae* var. *saké* is most abundant among other various kinds of fungi.

(3) When the *Aspergillus oryzae* var. *saké* is used for kōji-making, the heat radiation in culture is most abundant.

(4) When the kōji made by *Aspergillus oryzae* var. *saké* is soaked in water, the dissolved oxygen in the filtrate evidently decreases, therefore the alcohol fermentation by yeast takes place quickly.

Studies on the Microbiological Assay of Vitamins and Amino Acids by the Pulp Disc Method. Part III. On the Standard Curves for B-vitamins and Amino Acid Assay by the Pulp Disc Method. (*P. 100~106*)

By Hisao KOJIMA and Yataka MATSUYA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

Application of the pulp disc method to assay the members of B-vitamin previously not tested and also amino acids was attempted. Assay standard curve for eleven members of B-vitamin and eighteen amino acids by this method was made under the procedure conditions presented in the previous reports. The B-vitamins and amino acids tested were: thiamine, riboflavin, pyridoxine, nicotinic acid, pantothenate, biotin, folic acid, inositol, choline, *p*-aminobenzoic acid, vitamin B_{12}, DL-alanine, L-arginine, DL-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, DL-isoleucine, L-leucine, L-lysine, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine and DL-valine. Except *p*-aminobenzoic acid, a linear co-relationship was observed between the diameter of growth zone obtained and concentration of these vitamins and amino acids tested. In the test of several kinds of amino acid such as threonine, glycine and valine, sufficient sharply defined growth zones of the assay organism were frequently hardly observed. This may mainly be due to an inadequate adoption of the assay organism to this pulp disc method, because the interchange of the assay organism afforded much improvement in producing a well-discernible growth zone by this method. Furthermore, assay range of the vitamins as well as amino acids when tested are described in detail.

Studies on the Microbiological Assay of Vitamins and Amino Acids by the Pulp Disc Method. Part IV. On the Assay of Several Amino Acids by the Pulp Disc Method. (*P. 106～110*)

By Yutaka MATSUYA and Hisao KOJIMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

The pulp disc method for assay of L-lysine, DL-methionine, L-glutamic acid and DL-phenylalanine using *Lact. arabinosus* and *Leuc. mesenteroides* was especially examined in order to determine procedure conditions for its application. The assay range of these amino acids was also wider than that obtained by the tube method, where the relations between inoculum density, culture period and growth zone were further examined, and a comparison of this method with the ordinal tube method was made. The tested samples were yeast extracts, polypeptone, acid-hydrolysate of polypeptone, and casamino acid. The relationship between the concentration of the samples and diameter of growth zone obtained was quite linear, and the gradient of the line thus obtained for each of the above amino acids contained in the tested samples was the same as that obtained with each of their authentic preparation. Reproducibility of assay results was obtained in the range of 90–110%. The known amount of amino acid added to the samples was recovered in 95–105%. The values obtained by the two methods a satisfactory coincidence with each other, although it may be said that the values obtained by this method were, in general, slightly lower than those obtained by the tube method. NaCl, even when contained in the samples up to the concentration of 20%, did not affect the assay results.

Studies on the Formation Mechanism of Black Tea Aroma. Part III. Qualitative Experiments on the Volatile Carbonyl Compounds. (*P.110～114*)

By Toshio NAKABAYASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

In order to make clear the qualitative difference of the volatile carbonyl compounds (VCC) of black tea having either good or bad aroma, the ultra violet absorption spectra of the 2,4-dinitrophenylhydrazone of VCC (VCC-hydrazone), which were obtained by distilling the essential oil into 2,4-dinitro phenylhydrazine solution, were studied.

As the spectra of the obtained VCC-hydrazone were on the whole, all similar and that of good aroma had several sharp absorption peaks as compared with the easy spectrum of bad aroma, it was supposed that the constituents of VCC were all the same qualitatively being merely different in quantity, i.e., VCC of good aroma was little and had few components but that of bad aroma was much and had many components.

When the VCC-hydrazone was made alkaline by KOH, the color of VCC-hydrazone of good aroma turned to purple and that of bad aroma to red. By the investigation of the solubility in petroleum benzine and column chromatography, it was supposed that the petroleum benzine soluble-fraction of VCC-hydrazone consisted with i-valer, benz, perilla aldehyde and $\alpha.\beta$-hexenal and its color turns to red with alkali and with the petroleum benzine insoluble-fraction, from which six unknown components were obtained, turns to purple consisting of the hydrazone of ketone likely presumed.

Degumming for Waste Silk. Part VIII. Purification of Protease Produced by an Aerobic Bacteria (*P. 115～118*)

By Toshio NAKAHAMA and Hirotsugu IMAHARA

(*Department of Agriculture, Saikyo University*)

A useful strain of *Bacillus vulgatus* was cultured in peptone water with glucose for five days and the cultural filtrate was salted out by ammonium sulfate on the condition of 0.4 to 0.8 saturation. The salting matter was collected by centrifuge at 3,000 r.p.m. and dissolved in water. The solution was added acetone from 45 to 80% and then the precipitate was collected by centrifuge at 3,000 r.p.m. The above process of salting and precipitating was repeated. The final purifying matter was dissolved in water and acetone was added to 55% in the solution at 0°C and then crystals were formed in the solution. The form of the crystal protease was needle-shaped. The gelatine liquefying power of the crystal protease was 11.6 per 1 mg. and the optimum pH of the power was found to lie 7.8 and 8.2. The main component of the crystal was protein. Purity of the crystal was in vestigated by the Tiselius-electrophoresis method. The decomposing power for casein and gelatine was almost the same as trypsine. But the specific characteristic of the crystal protease is that this enzyme decomposes more sericine than trypsine.

Effects of Antibiotics on Contaminants of Sake-moromi mash. (*P. 119～124*)

By Shin SATO

(*Appraisal Official's Room, Tokyo Tax Administration Bureau, Ministry of Finance*)

The application of antibiotics for the control of bacterial infection in Saké-Moromi fermentation is the subject of this investigation.

Minimal inhibitory concentrations of eleven antibiotics have been determined for certain species of lactobacillus: streptococcus, pediococcus, leuconostoc, acetobacter and gluconobacter, which are supposed to be contaminants of Saké-Moromi mash.

Aureomycin, terramycin and achromycin were found to be sufficiently active against all species tested. All gram-negative bacteria tested, showed resistance to penicillin at 20 γ per ml, a concentration which inhibited most of the species of gram-positive bacteria, and the converse holds good.

But polymyxin, colistin, neomycin which were reported as effective antibiotics for brewery contaminants by other authors, proved not active against almost all the species tested.

Besides, it was ascertained that the rate of fermentation by Saké-yeast and the taste of produced Saké are not affected by high concentrations of penicillin and streptomycin, and hardly affected by high concentrations of aureomycin, achromycin, terramycin and chloromycetin.

Consequently, it was concluded that aureomycin, achromycin, terramycin or penicillin+streptomycin in the range of 4~20 γ per ml are sufficiently active to be practical for the purpose of controlling bacterial infections of Saké-Moromi fermentations.

Studies on the Inhibitory Factors in Acetone-butanol Fermentation. Part I. The Features of Abnormal Fermentation. (*P. 124~128*)

By Shigeru NEMOTO
(*Godo-Shusei Inc.*)

From the three standpoints, observational, analytical, and morphological, the author has studied the abnormalities resulting from the so-called "sleeping sickness" in acetone-butanol fermentation.

This type of abnormal fermentation can belonged to sluggish fermentation. The elongation in time is chiefly due to the retardation in growth of bacteria. Abuot 30–40 hours are extended in total.

During this retardation in growth of bacteria, gas ceases to evolve for a certain time, and then, begins to evolve again.

A sluggish increase in acidity and a sluggish fall of pH are the characteristics of this sleeping-period. Durihg this period, the bacilli are very few in number, and abnormal in form. These bacilli (6μ~10μ×0.3μ~0.5μ) are long and thin due to thin due to their side breaking down.

The othdr bacilli (3μ~4×0.8μ) co-exists during this period. A little while after that, the bacilli form a long linkage (30μ to 50μ).

At the end of fermentation, the long linkage of the bacilli appears out with the bacilli shorter in size once again.

Solvent formation is slow, but is not different from normal formation in a productive amount. However, the distillation in ratio is a little lower than that of normal solvent formation.

Studies on the Inhibitory Factors in Acetone-butanol Fermentation. Part II. Investigation of the Condition for Fermentation which are supposed to cause Abnormal Fermentation. (*P. 128~132*)

By Shigeru NEMOTO
(*Godo-Shusei Inc.*)

The author has studied the factors which were supposed to be the cause of abormal fermentation. This abnormal fermentation could not be stopped by changing all the strains of butanol bacilli available. In the case of over-cooking a sluggish fermentation occured. However, he could not come to the conclusion that this is the chief cause of this abnormal fermentation.

The broth from the various step of fermentation of industrial tanks were removed in small flasks, and fermentation of these flasks was investigated. The flasks sampled just at the time of gas evolution or just after inoculation when the number of normal butanol bacilli were large, continued to fermentate. But the flasks sampled at the stage when gas evolution stopped and those sampled when the number of the bacilli were small, did not start fermentation again, though in the large fermenter fermentation took place again.

As a result of these studies on the effect of nutritious substances on fermentation, he has found that Vitamin B_1, Metabolin, and K_2HPO_4 are effective.

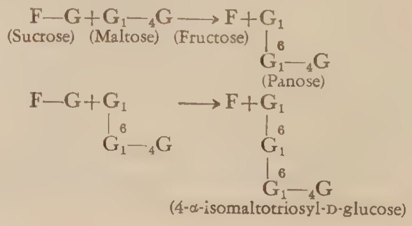
Synthesis of Oligosaccharides by a Growing Culture of *Leuconostoc mesenteroides*. Part I. Oligosaccharides formed from Reaction of Sucrose and Glucose-Polymers. (*P. 133~138*)

By Kazuo SHIBASAKI
(*Faculty of Agriculture, Tohoku University*)

It has been shown that oligosaccharides are synthesized by the growing culture of *Leuconostoc mesenteroides* on sucrose in the presence of certain simple sugars, and dextran production was noticeably depressed. Paper chromatographic evidence was obtained of: (a) isomaltose, isomaltotriose on the addition of glucose to the culture medium: (b) panose, 4-α-isomaltotriosyl-D-glucose, 4-α-isomaltotetraosyl-D-glucose on the addition of maltose: (c) isomaltotriose, isomaltotetraose and their higher homologues on the addition of isomaltose.

The oligosaccharides formed from the reaction of sucrose and maltose were isolated by chromatography on a charcoal-celite column.

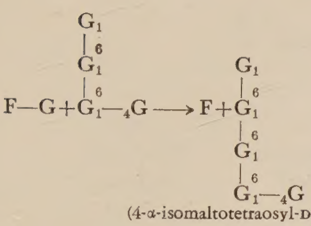
The reaction mechanism has been suggested to be as follows:



(1) $$\underset{\text{(Sucrose)}}{\mathrm{F{-}G}} + \underset{\text{(Maltose)}}{\mathrm{G_1{-}_4G}} \longrightarrow \underset{\text{(Fructose)}}{\mathrm{F}} + \underset{\text{(Panose)}}{\begin{array}{l}\mathrm{G_1}\\ |\,{}_6\\ \mathrm{G_1{-}_4G}\end{array}}$$

(2) $$\mathrm{F{-}G} + \begin{array}{l}\mathrm{G_1}\\ |\,{}_6\\ \mathrm{G_1{-}_4G}\end{array} \longrightarrow \mathrm{F} + \underset{\text{(4-α-isomaltotriosyl-D-glucose)}}{\begin{array}{l}\mathrm{G_1}\\ |\,{}_6\\ \mathrm{G_1}\\ |\,{}_6\\ \mathrm{G_1{-}_4G}\end{array}}$$

(3)



$$F-G+\begin{array}{c}G_1\\|_6\\G_1\\|_6\\G_1-_4G\end{array}\longrightarrow F+\begin{array}{c}G_1\\|_6\\G_1\\|_6\\G_1\\|_6\\G_1-_4G\end{array}$$

(4-α-isomaltotetraosyl-D-glucose)

Studies on the Analytical Methods of Soy Sauce. Part I. The Estimation of Glycerin in Soy Sauce. (*P. 138~143*)

By Akira OKUHARA and Tamotsu YOKOTSUKA
(*Noda Soy Sauce Co. Ltd.*)

Analytical methods for the separation of glycerin from other ingredients in soy sauce, especially from mannitol, were studied. Distillation in vacuum, which was performed by adding a rather great amount of water to the sample for several times, gave good results, exclusively in comparision with the other methods, including the extraction of glycerin by some adequate solvents, precipitating off the other ingredients, and steam distillation in vacuum. The isolated glycerin was determined colorimetrically, after being oxidized by periodicacid. The content of glycerin in marketing soy sauces was about 0.5–1.2%.

Studies on the Analytical Methods of Soy Sauce. Part II. Potentiometric Titration of Acidic Ingredients and Formol Nitrogen of Soy Sauce.(*P. 144~151*)

By Toshio SAKASAI and Tamotsu YOKOTSUKA
(*Noda Soy Sauce Co. Ltd.*)

Good results were obtained when the acidic content of soy sauce was determined by potentiometric titration at the end-point of pH 8.2. Formol nitrogen was recovered in almost 100%, when the mixture of soy sauce, which was adjusted at pH 8.5, and formalin of pH 8.5 was potentiometrically titrated till pH 8.5 was obtained. In this case the final concentration of formalin should be 9–15%.

Butanol Fermentation. Part XVII. Redox Potential Studies on the Shortening of Fermentation Period by Successive Subculturing. (*P. 155~158.*)

By Motoyoshi HONGO
(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

In the fermentation of sucrose by *Clostridium acetobutylicum* 314 in a medium containing soy-bean cake as the nitrogen source, the time required to complete fermentation is shortened by several continuous transplantations of seed culture and prolonged by its further transplantations. Some factors causing this change of fermentation-period were investigated resulting as follows:

1. The substances poising the medium in a low potential level (liver, flavine or neutral red) accelerated the growth of this bacteria, but did not accelerate the fermentation except flavin. Flavin had little effect for acceleration of the fermentation.
2. In the each culture inoculated by strong seed culture tronsferred one time from the culture heat-shocked or inoculated by weak seed culture transferred seven times, courses of the changes of rH, pH, acidity, and fermenting velocity were studied. It was ascertained that the position of the rH curve to the acidity curve changed by the weakening of culture.

On the basis of the above results and others previously reported1) the causes for shortening and prolongation of the fermentation period by successive subculturing in various physiological stages are discussed.

Denaturation of Lysozyme by Ultraviolet Light. (*P. 158~161*)

By Yataro OBATA and Mitsuo KOSHIKA
(*Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo*)

An egg-white lysozyme solution was put in a vessel covered with quartz glass and irradiated with ultraviolet light. The lytic activity of lysozyme was decreased, and the inactivated enzyme was not reactivated by addition of cystein and hydrogen sulfide. The ultraviolet absorption spectrum was also changed by this irradiation, especially, decrement of the peak at 280 mμ was remarkable. No changes in tyrosine and histidine contents of lysozyme could be observed, but about half of the tryptophan content was destroyed when the enzyme activity entirely disappeared. The decrease of the extinction coefficient of the absorption maximum was due to the destruction of tryptophan.

1) Nippon Nogei-Kagaku Kaishi, **30**, 729, 758 (1956).

Additions and Corrections

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A DELEGATION FROM AGRICULTURAL CHEMICAL FIELD OF JAPAN: Report of Inspection Tour in People's Republic of China. (2)

Technical Article

T. KOYAMA: To-days Cosmetic Industry in Japan.

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